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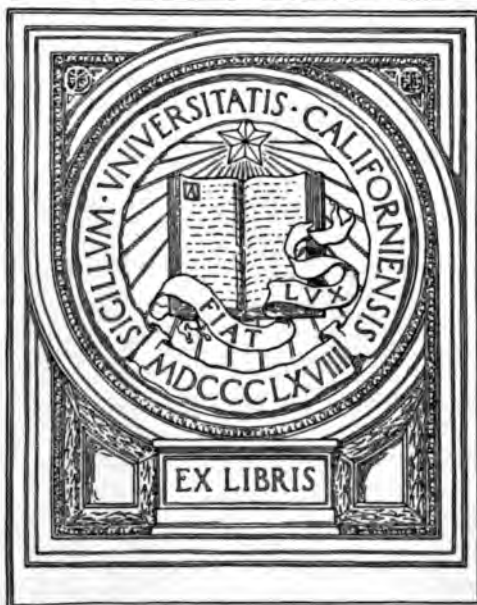


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CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM AS RELATED TO MILK SECRETION.*

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(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture.)

(Received for publication, September 26, 1918.)

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2 Phosphorus and Calcium Metabolism

INTRODUCTION.

For the development of our knowledge of the physiology of milk secretion, it is desirable that we should discover the precursors in the blood of the various constituents of milk. Very considerable progress has already been made in this field; it seems to us to be peculiarly accessible to experimental investigation, and we have devoted a good deal of time to working in it.

When the precursors of the milk constituents are known, it will be desirable to discover how their concentration in the blood is maintained against the great demand made for them by the mammary glands of heavily milking animals. We believe that light can be thrown on this subject by studying the concentration of certain materials in the blood under various conditions, and we have made a more or less extended study along these lines. We have paid particular attention to phosphorus and calcium, but we have studied also other constituents of the blood, when this promised further light on the problems in hand.

EXPERIMENTAL METHODS.

Our experiments have consisted in analyzing the blood and plasma obtained from the jugular or from the abdominal subcutaneous vein of intact normal or nearly normal cattle. The blood samples were obtained by means of a small trochar and cannula inserted through the skin into the vein. In expert hands the operation is an extremely simple one; it has never, in our experience, had any perceptible effect on the health or on the milk yield of the animals subjected to it. Our only antiseptic precaution has been to wash the skin and hair of the subject near the seat of operation, as well as the trochar and cannula, with 5 per cent carbolic acid solution.

The details of our experimental procedures have naturally varied from time to time. In describing them it will be most convenient to lay the chief emphasis on the routine which we consider the best at the present time, and later to add remarks on the changes that we have made in cases where this seems to be worth while.

There has not been, up to the present time, very much work done on the variations which occur in the concentration of the ash constituents of tissues, and particularly of the calcium and phosphorus of the blood, under normal conditions. In such work it is desirable to make a great many analyses and to be able to detect even small variations. In addition, a long experiment may often be rendered completely valueless or even positively misleading, if a single pair of incorrect results is obtained at some

critical point in its course. In beginning such work, therefore, it is important to develop methods of the highest possible accuracy, certainty, and rapidity.

We have tried a number of the methods recommended in the standard text-books and found them unsatisfactory; and the methods which we now use have been developed by a process of selection, and as the result of considerable experience. We think it advisable, therefore, to report them in some detail.

Collection of Blood; Prevention of Coagulation.

Our blood samples have been collected in clean glass bottles in which had previously been placed some reagent to prevent clotting. When we wish to analyze for phosphorus only, sodium oxalate is placed in the bottle in such quantity that each 100 cc. of blood collected will be mixed with 0.1 gm. of the salt; where calcium analyses are to be made, 1 cc. of saturated sodium citrate solution is mixed with each 100 cc. of blood. It is, of course, impossible to use plasma from oxalated blood for calcium analyses, as most of the plasma calcium is precipitated by the oxalate, and thrown down with the corpuscles during the centrifuging.

We have been able to show that oxalate does not interfere with phosphorus analyses as carried out by our methods and that citrate does not interfere with analyses for either calcium or phosphorus. We found it practicable to prevent clotting by drawing our blood into cooled vessels lined with paraffin and by keeping the blood and plasma in cooled paraffined vessels during the centrifuging and until we were ready to use it. In still other instances we prevented clotting by the use of hirudin. The blood and plasma kept fluid by the first of these methods may be called "natural" blood and plasma. It was found that if different portions of the same sample of blood were kept fluid by the "natural" method, by hirudin, and by citrate, the portions of plasma obtained from them all gave similar results for calcium and phosphorus; and that the portions of plasma obtained from "natural," hirudinized, citrated, and oxalated blood all gave similar results for phosphorus.

Centrifugation of Blood.

Our blood samples have been centrifuged, as a matter of routine, for 20 minutes at between 3,000 and 3,500 revolutions per minute, and in glass vessels marked so that the volume of the corpuscles could be read off when the centrifugation was finished. We found that longer centrifuging at higher speeds did not reduce the volume of the corpuscles any further.

Solutions and Reagents.

We have been in doubt as to what is meant in text-books of chemistry when one is told to make up a 10 per cent solution or a 25 per cent solution. Our procedures may, therefore, occasionally be somewhat differ-

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ent in detail from what was intended in the descriptions from which we have taken them. But, as a preliminary step to adopting any method of analysis, we have naturally carried out a number of check analyses on known amounts of chemically pure substances; and, if our results were not satisfactory at first, we have varied our procedure until they became so. In describing our own solutions, we shall speak generally in terms of solutions by weight. By a "10 per cent hydrochloric acid solution," for example, we mean a solution of which 100 gm. contain 10 gm. of HCl and 90 gm. of water. In the case of salts which contain water of crystallization we shall give the strengths of our solutions in terms of the water-free salt.

Method of Ashing Material.

We have analyzed our samples of blood and plasma for calcium, total phosphorus, lipid and inorganic phosphorus, and nitrogen, and in a few cases for total fat.

For the calcium and total phosphorus analyses the blood or plasma has been ashed with acid mixture, essentially as recommended by Abderhalden.¹ When this procedure is carried out with plasma, the concentrated sulfuric acid ash is always quite clear and colorless; in the case of blood, on the other hand, the concentrated ash contains a whitish precipitate, which dissolves in water and gives a positive test with potassium sulfoeyanate, and which, we have no doubt, is ferric sulfate. It dissolves when the acid ash is boiled with water, leaving a very faintly yellow color; and it does not interfere with the calcium and phosphorus analyses.

Determination of Calcium.

Our method of determining the calcium in blood and plasma consists in a combination of parts of the procedure recommended by Abderhalden² with parts of McCrudden's method.³ 30 to 50 cc. of blood or plasma are ashed with 25 cc. of acid mixture,⁴ and the acid ash is finally boiled with about 25 cc. of water. The mixture is then transferred with the aid of a little water from the Kjeldahl flask used for ashing to a beaker, five times the volume of the mixture and washings in 95 per cent alcohol is added, and the whole is allowed to stand over night. The calcium sulfate so precipitated is filtered off, and washed three or four times with 95 per cent alcohol; the filtrate and washings may be used for the determination of total phosphorus (see below).

The filter paper with the calcium sulfate precipitate is dried and ashed in a platinum crucible and dissolved in approximately 35 per cent HCl by

¹ Abderhalden, E., *Handb. biochem. Arbeitsmethoden*, Berlin, 1910, i, 385-388.

² Abderhalden, *Handb. biochem. Arbeitsmethoden*, Berlin, 1910, i, 413.

³ McCrudden, F. H., *J. Biol. Chem.*, 1911-12, x, 187.

⁴ Equal parts by volume of 95 per cent H₂SO₄ and 70 per cent HNO₃.

digestion for 45 minutes at from 50–75°. The HCl solution so obtained is diluted with water, filtered into a beaker, and made up to about 75 cc. with water. One drop of 0.006 per cent phenolsulfonephthalein is added, and then 28 per cent NH_3 until the color changes through yellow to pink. The mixture is cooled and 10 per cent HCl is added drop by drop until the color changes back to clear yellow. 10 cc. of 0.5 N HCl and 10 cc. of 1.75 per cent $\text{H}_2\text{C}_2\text{O}_4$ are then added; the mixture is heated to boiling, then cooled to 50° or below; 10 cc. of 10 per cent $\text{NaC}_2\text{H}_3\text{O}_2$ are added, and the mixture is allowed to stand about 30 minutes at between 50 and 75°, and then over night at room temperature. It is filtered through a small filter paper, and the precipitate is washed free of chlorides with 0.4 per cent $(\text{NH}_4)_2\text{C}_2\text{O}_4$, and then washed free of soluble oxalate with water. Finally it is dissolved in about 20 cc. of warm approximately 2 N H_2SO_4 , and washed through the filter with water to about 100 cc. It is heated to 70° and immediately titrated with 0.02 N KMnO_4 , the calcium being calculated from the amount of standard permanganate solution required.

We have standardized our permanganate solutions frequently against known oxalate solutions. We found, however, that if the permanganate is carefully made up and kept in clean glass vessels, its strength does not change very much even in several months.

Abderhalden recommends weighing the calcium sulfate from the acid ash, either after one⁵ or after two⁶ precipitations with alcohol. We tried both these procedures and found them highly unsatisfactory for blood and rather unsatisfactory for plasma. We also tried various other methods of calcium analysis, such as drying our material and ashing it in platinum over a flame, and weighing the calcium as oxide or oxalate. These methods are, of course, capable of giving accurate results, but we found them decidedly more troublesome than the procedure described above, which is satisfactory both for blood and plasma.

Determination of Total Phosphorus.

When we wished to determine the calcium and phosphorus in the same sample of material, the alcoholic filtrate from the calcium sulfate precipitation (see above) was placed in a Kjeldahl flask and evaporated on the steam bath until the alcohol was nearly or completely driven off.⁶ The concentrated filtrate was boiled until it became dark, and 5 cc. of 70 per cent HNO_3 were allowed to flow into it drop by drop while it was still boiling. The boiling was continued for about 20 minutes more, and the sulfuric acid ash so obtained was treated as described below.

When phosphorus alone was to be determined, the sample (20 to 35 cc. of blood or 30 to 50 cc. of plasma) was ashed with 25 cc. of acid mixture in

⁵ Abderhalden, *Handb. biochem. Arbeitsmethoden*, Berlin, 1912, vi, 381.

⁶ Evaporation from a flask may be very much hastened by inserting a glass tube down its neck nearly to the surface of the liquid to be evaporated, and sucking air through this by means of the vacuum pump.

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the same manner as for the calcium determination. The sulfuric acid ash so obtained may be regarded as equivalent, so far as phosphorus is concerned, to the ash obtained from the alcoholic filtrate of the calcium determination, as described in the preceding paragraph, and from this point the description applies to both.

The concentrated sulfuric acid ash is boiled with about 30 cc. of water for a few minutes and then transferred to a beaker and diluted with water to 150 cc. 50 cc. of 33 per cent NH_4NO_3 are added, and the mixture is heated to boiling. At the same time 40 cc. of 8.6 per cent $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ are brought to boiling and poured into the phosphorus mixture. The resulting precipitation is again brought to a boil and stirred about 2 minutes; it is then allowed to stand 10 minutes or as much longer as may be convenient.⁷

The ammonium phosphomolybdate is filtered by decantation and washed once with 25 cc. of wash liquid;⁸ then dissolved in 5 cc. of 10 per cent NH_3 ; 10 cc. of 25 per cent NH_4NO_3 , 15 cc. of water, and 1 cc. of 2.7 per cent $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ are added, the mixture is brought to boiling, and reprecipitated by the addition of 10 cc. of boiling 31 per cent HNO_3 ; it is stirred for 2 minutes and then allowed to stand 10 minutes or more. It is again filtered by decantation, and the precipitate is dissolved without further washing in 12 cc. of 2.5 per cent NH_3 , to which about 20 cc. of water are added. To the resulting solution 35 per cent HCl is added drop by drop until the precipitate resulting from one of the drops dissolves only slowly on stirring, and there are then added 2 cc. of magnesia mixture⁹ and one drop of 1 per cent phenolphthalein. The solution is brought to a boil and 2.5 per cent NH_3 is added quickly from a pipette until a faint pink color appears; it usually requires 5 to 10 cc. The mixture is cooled to somewhat below room temperature, as a result of which numerous crystals of MgNH_4PO_4 should appear. About 10 cc. of 28 per cent NH_3 are added, and the mixture is allowed to stand 10 minutes more, or as much longer as may be convenient. It is filtered through a weighed Gooch crucible, the precipitate washed free of chlorides with 2.5 per cent NH_3 , dried, and ignited to $\text{Mg}_2\text{P}_2\text{O}_7$; and the phosphorus is calculated from the weight of the pyrophosphate.

⁷ The part of the procedure so far described is, in general, taken from Abderhalden (Handb. biochem. Arbeitsmethoden, Berlin, 1910, i, 420). Abderhalden, however, recommends heating the ammonium phosphomolybdate precipitation only to 70–80°, while we found that the phosphorus did not all come down unless it was heated to boiling as described above. Abderhalden recommends carrying the analysis further by the titration method, which we tried and found not entirely satisfactory. From this point in our analysis, we followed in general the procedure recommended by Treadwell (Analytical chemistry, New York 2nd edition, 1910, ii, 397–398).

⁸ NH_4NO_3 , 100 gm.; 70 per cent HNO_3 , 80 cc.; water, 2 liters.

⁹ $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 110 gm.; NH_4Cl , 210 gm.; 35 per cent HCl , 4 cc.; water, 2 liters.

Determination of Inorganic Phosphorus.

The inorganic phosphate of plasma has usually been determined by a method developed by one of us¹⁰ from a brief description given by Greenwald.¹¹

To 80 cc. of plasma are added 160 cc. of water, 80 drops 0.1 N HCl, 4 cc. of saturated NH_4Cl , 1.6 cc. of 7.7 per cent MgCl_2 , 32 cc. of 2.8 per cent NH_3 , and the mixture is allowed to stand over night, preferably at about 5°C. If smaller quantities of plasma are taken, all the above mentioned reagents are reduced proportionally. The mixture is filtered, and the precipitate is washed three or four times with 2.5 per cent NH_3 , twice with 95 per cent alcohol, and twice with ether. It is then dissolved from the filter paper into a beaker with 15 cc. of 8 per cent HNO_3 , 10 cc. of 31 per cent HNO_3 , and 45 cc. of water; 10 cc. of 25 per cent NH_4NO_3 , and 30 cc. of 2.7 per cent $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ are added, the mixture is heated for an hour or so to from 50-75°, and then allowed to stand over night at room temperature. (The precipitate will come down completely without heating, if allowed to stand 48 hours at room temperature.) The mixture is filtered by decantation, and the analysis is carried to completion exactly as in the case of the determination of total phosphorus after the filtration of the second ammonium phosphomolybdate precipitation (see above).

In a few instances we determined the inorganic phosphate of plasma by the method described by Embden and his collaborators¹² for muscle juice. The same samples of plasma were analyzed by this method and by the one which has just been described and the results are given below.

Inorganic Phosphate in Plasma. Mg. per 100 Gm. of Plasma.

Plasma.	Determination.	Greenwald method.	Embden method.
		mg.	mg.
1	I	4.26	4.49
	II	4.30	4.49
	Average.....	4.28	4.49
2	I	5.89	6.07
	II	5.80	6.30
	Average.....	5.84	6.18
3	I	3.72	4.13
	II	3.99	3.96
	Average.....	3.85	4.04
4	I	4.66	4.85
	II	4.80	4.61
	Average.....	4.73	4.73

¹⁰ N. R. Blatherwick.

¹¹ Greenwald, I., *J. Biol. Chem.*, 1916, xxv, 431.

¹² Embden, G., Griesbach, W., and Schmitz, F., *Z. physiol. Chem.*, 1914-15, xciii, 1.

In two other cases the plasma was analyzed immediately by the Greenwald method and after it had stood for some time, by the Embden method. The results were as follows:

	Greenwald method.	Embden method.
	mg.	mg.
Plasma 5. Analyzed by Embden method after standing 4 days.	I 6.34 II 6.25 Average. 6.29	7.00 6.91 6.95
Plasma 6. Analyzed by Embden method after standing 1 day.	I 4.13 II 3.89 Average. 4.01	4.40 4.66 4.53

For the first four samples of plasma the figures obtained by the Embden method average 0.18 mg. per 100 gm. of plasma higher than those obtained by the Greenwald method. This difference is very small; the question of its significance will be discussed later.

In the last two samples of plasma, which were analyzed immediately by the Greenwald method, and not until after standing some time by the Embden method, the differences in the figures obtained by the two methods are greater than in the other four. These results indicate that the phosphatide of the plasma tends to decompose somewhat on standing; and that, in cases where the highest accuracy is desired, the samples should be analyzed immediately.

Determination of Lipoid Phosphorus.

The great majority of our determinations of lipoid phosphorus have been carried out by the nephelometric micro method, which was suggested some time ago by Pouget and Chouchak,¹³ and has since been developed by Greenwald,¹⁴ Kober,¹⁵ and Bloor.¹⁶ A more or less detailed account of our experience with this method has already been published.¹⁷

We tested the micro method of phosphorus determination in several ways. Parallel determinations were carried out on the same samples of blood and plasma by the gross method described above and by the micro method. For the latter determination about 0.2 gm. of blood or 0.5 gm. of plasma was ashed with 1.5 cc. of acid mixture and the analysis was

¹³ Pouget, I., and Chouchak, D., *Bull. Soc. chim. France*, 1909, v, 104; 1911, ix, 649.

¹⁴ Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

¹⁵ Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

¹⁶ Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133, 145.

¹⁷ Meigs, E. B., *J. Biol. Chem.*, 1918, xxxvi, 335.

carried further in general as recommended by Bloor.¹⁸ The results were as follows:

Total Phosphorus in Blood and Plasma. Mg. per 100 Gm.

	Micro method.		Gross method.	
		mg.		mg.
Blood.....		17.94	I	18.55
			II	18.51
			Average.....	18.53
Plasma 1.....		12.80	I	13.09
			II	13.70
			Average.....	13.39
Plasma 2.....	I	12.38		
	II	12.07		11.51
	Average.....	12.22		

In other samples of plasma parallel determinations were made of the lipid phosphorus by the micro method and by the gross method described by Koch and Woods.¹⁸ The results were as follows:

Lipid Phosphorus in Plasma. Mg. per 100 Gm.

Plasma.	Micro method.	Gross method.
	mg.	mg.
1	7.1	6.8
2	5.0	4.5
3	7.1	6.7
4	7.0	6.5
5	6.4	5.9

We carried out similar parallel determinations on three other samples of plasma, in which the two sets of results did not agree at all; these were among our earliest determinations by the micro method, and we have every reason to believe that the results were incorrect. We do not think it worth while to give the figures for these determinations, and they are mentioned only because this case forms an exception to our rule of giving all the results obtained bearing on any particular point.

It will be seen that the results obtained by the nephelometric method run about 0.4 mg. per 100 gm. higher than do the Koch and Woods results. Following these experiments, we extracted gross samples of plasma with

¹⁸ Koch, W., and Woods, H. S., *J. Biol. Chem.*, 1905-06, i, 203.

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alcohol and ether, just as we had done to obtain the Koch and Woods results given above, and then extracted the same samples further, and found that there was additional lipid in the subsequent extractions. So far as we know, it has been the universal experience of those who have tried to extract lipid material by alcohol and ether from such large quantities of protein as one gets in gross samples of plasma that the lipid is not completely extracted in any reasonable time. We agree with Bloor, therefore, that the micro-extraction described by him is decidedly more complete and satisfactory than even very long drawn out gross extractions; and the results given above indicate that our nephelometric results for lipid phosphorus in plasma are very nearly correct.

Determination of Total Fat.

Fat has been determined in blood and plasma according to the Bloor method and the later modifications suggested by him.¹⁹ This procedure was tried against the well known Babcock method on samples of milk, and was found to give results within 5 per cent of those given by the Babcock method.

Determination of Nitrogen.

In determining nitrogen we have used the Kjeldahl method described by Abderhalden.²⁰ We encountered no difficulties in following the procedure.

Nature of the Phosphorus Compounds Contained in Normal Plasma.

Abderhalden recognizes the existence of three classes of phosphorus compounds in blood serum: (1) inorganic phosphate; (2) phosphorus combined as lecithin; (3) phosphorus combined as nuclein. He finds in ox serum 0.0037 per cent inorganic phosphorus, 0.0067 per cent lecithin phosphorus, and 0.0006 per cent nuclein phosphorus, making a total of 0.0110 per cent.²¹ He has analyzed the serum of a number of other domesticated mammals, and finds very similar figures.

¹⁹ Bloor, *J. Biol. Chem.*, 1914, xvii, 377 ff.; 1915, xxiii, 320.

²⁰ Abderhalden, *Handb. biochem. Arbeitsmethoden*, Berlin, 1910, i, 340 ff.

²¹ Abderhalden, *Z. physiol. Chem.*, 1897, xxiii, 521; 1898, xxv, 65. The "nuclein" and inorganic phosphorus is given by this author as P_2O_5 ; and the amount of "lecithin" phosphorus found is not given. The figures which appear above are for elemental phosphorus; those for "nuclein" and inorganic phosphorus are obtained by multiplying Abderhalden's figures by 0.44; those for "lecithin" phosphorus, by multiplying his figures for lecithin by 0.04.

Rona and Takahashi²² have recently had occasion to determine the different kinds of phosphorus compounds in horse serum. They find, as an average in seven experiments, 0.0005 per cent nuclein phosphorus.

Other work directed more particularly toward the determination of nuclein or protein phosphorus in blood serum makes it seem probable that the figures given by Abderhalden and by Rona and Takahashi are too high. Liebermeister,²³ for instance has endeavored to separate the nucleoprotein from serum and gives figures for the amount of material obtained and for its phosphorus content. He got only 2.5 gm. of nucleoprotein from 15 liters of serum; the phosphorus content of this material was 0.079 per cent. This would indicate only 0.00001 per cent nuclein phosphorus in the whole serum; even if it is supposed that Liebermeister got less than half of the total quantity of nucleoprotein present, his work would still indicate that the nuclein phosphorus content of serum is only a small fraction of that given by Abderhalden and by Rona and Takahashi.

Even the figures of these latter authors are small—about 5 per cent of the total phosphorus contained in serum. Abderhalden gives only a very brief description of his methods; Rona and Takahashi, none at all of theirs; so that one is not able to form a critical judgment as to the correctness of their determination of the nuclein phosphorus.

The evidence so far given, therefore, indicates that there cannot be more than about 0.5 mg. of nuclein phosphorus in 100 gm. of serum, and that there is probably much less than this.

It has been shown by Bürker²⁴ that adding oxalate to blood prevents the breaking up of the blood platelets, which occurs when it is allowed to clot; when oxalated blood is centrifuged these elements are thrown down at the top of the layer of corpuscles. The plasma from oxalated blood, therefore, differs from serum in that it does not contain the break-down products of these cellular elements. The blood platelets probably contain nucleoprotein;²⁵

²² Rona, P., and Takahashi, D., *Biochem. Z.*, 1913, xlix, 370.

²³ Liebermeister, G., *Beitr. chem. Physiol. u. Path.*, 1906, viii, 439.

²⁴ Bürker, K., *Arch. ges. Physiol.*, 1904, cii, 88.

²⁵ Deetjen, H., *Z. physiol. Chem.*, 1909, lxiii, 1. Deetjen has demonstrated that the blood platelets contain nuclei.

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and one would, therefore, expect to find less nuclein phosphorus in oxalated plasma than in serum.

In 47 samples of plasma we have made independent determinations of the lipoid, inorganic, and total phosphorus by the methods described above. The results are given in Table I.

The figures in Table I for lipoid, inorganic, and total phosphorus are based on duplicate determinations which agreed within 5 per cent, except as indicated in the footnotes.

Table I shows that the sum of the lipoid and inorganic phosphorus fractions is generally a little lower than the total as determined independently, the difference amounting on the average to 0.26 mg. per 100 gm. of plasma. If the figures for the individual analyses are examined, it will be seen that the individual differences do not vary much from this average. In 37 out of the 47 cases they are not over 0.5 mg. per 100 gm. of plasma; in 9 cases they are over 0.5 mg. and not over 1.0 mg.; and in only 1 case is this difference above 1.0 mg.; namely, 1.7 mg.

In considering the results given in Table I, it is to be remembered that the limits of error for our analyses of total phosphorus in plasma are about 0.5 mg. per 100 gm. of plasma. It is to be remembered also that the analyses from which the figures were taken were, at one time or another, carried out by five different workers, and that as a rule the three analyses for a given sample of plasma were performed by three different workers. Further, each of the methods used for the determination of lipoid, inorganic, and total phosphorus respectively was widely different from either of the others, so that there would be little chance of constant errors in any two of the methods compensating for each other and so masking each other. It seems to us, therefore, that these results indicate fairly clearly that the lipoid and inorganic phosphorus of plasma comprise very nearly all that exists. Whether or not the fact that the sum of our figures for lipoid and inorganic phosphorus is, on the average, 0.26 mg. per 100 gm. lower than the total indicates that plasma contains a very small amount of some other kind of phosphorus, can be better discussed after considering Greenwald's work.

Greenwald has reached conclusions very similar to ours from his recent work on the phosphorus compounds of the blood. His work taken in connection with our own shows that there is no measurable quantity of nuclein phosphorus in plasma.

TABLE I.

*Relation between the Sum of the Lipoid and Inorganic Phosphorus Concentrations in Plasma, and the Total as Determined Independently.
Mg. per 100 Gm. of Plasma.*

No. of cow	Date.	Phosphorus.				
		Lipoid.	Inorganic.	Sum.	Total.	Difference.
		mg.	mg.	mg.	mg.	mg.
9	Dec. 15, 1916.....	5.3*	3.9	9.2	9.6	+0.4
	Jan. 17, 1918.....	4.8	6.3	11.1	10.8	-0.3
	Feb. 14, ".....	4.7	5.3	10.0	10.0	0.0
17	Nov. 1, 1917.....	6.0*	4.1	10.1	10.2	+0.1
19	" 7, 1916.....	8.5	5.9	14.4	13.9	-0.5
	Dec. 22, ".....	5.7*	5.0	10.7	11.1	+0.4
	Jan. 30, 1917.....	6.4*	6.0	12.4	12.4	0.0
	Mar. 9, ".....	5.0*	5.6	10.6	11.0	+0.4
	Apr. 9, ".....	7.3*	4.9	12.2	12.0	-0.2
	May 9, ".....	8.7	6.8	15.5	15.7	+0.2
	" 22, ".....	8.1	4.9†	13.0	13.3	+0.3
	June 22, ".....	8.7	4.6†	13.3	14.0	+0.7
27	Oct. 25, 1916.....	5.6	3.8	9.4	9.7	+0.3
	Dec. 8, ".....	6.4*	4.9	11.3	11.2	-0.1
	Feb. 12, 1917.....	6.2*	4.1	10.3	10.6	+0.3
33	Oct. 19, 1916.....	7.1*	5.4	12.5	12.7	+0.2
	Nov. 2, ".....	7.7†	4.6	12.3	13.2	+0.9
	Dec. 4, ".....	8.3*	5.4†	13.7	14.2†	+0.5
	Feb. 5, 1917.....	8.9*	4.3	13.2	13.4	+0.2
	Oct. 6, ".....	8.0*	4.2	12.2	12.5†	+0.3
39	" 16, 1916.....	6.4*	5.1	11.5	12.1	+0.6
	" 30, ".....	6.5	4.6†	11.1	11.2	+0.1
	Nov. 27, ".....	7.7	5.7	13.4	13.4	0.0
	Jan. 23, 1917.....	6.8*	4.6†	11.4	12.1	+0.7
	Mar. 26, ".....	6.4	5.3*	11.7	11.5	-0.2
79	Dec. 12, 1916.....	2.0*	6.3	8.3	8.5	+0.2
	Feb. 17, 1917.....	3.1*	6.4*	9.5	9.5†	0.0
80	" 10, ".....	3.1*	6.1	9.2	9.7†	+0.5
	Apr. 12, ".....	2.7*	8.7*	11.4	11.1	-0.3
81	Feb. 20, ".....	2.6	6.0†	8.6	9.6†	+1.0

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TABLE 1—*Continued.*

No. of cow.	Date.	Phosphorus.				
		Lipoid.	Inorganic.	Sum.	Total.	Difference.
		mg.	mg.	mg.	mg.	mg.
82	July 11, 1917.....	3.3	5.9*	9.2	10.1‡	+0.9
105	Dec. 19, 1916.....	6.3*	4.0	10.3	10.9	+0.6
	Feb. 24, 1917.....	7.2	4.7	11.9	12.6	+0.7
106	Jan. 4, 1918.....	4.9	4.6	9.5	9.5	0.0
107	Dec. 11, 1916.....	7.9*	5.2	13.1	13.4	+0.3
	Mar. 1, 1917.....	8.1*	4.8	12.9	13.2	+0.3
115	Nov. 10, 1916.....	6.1	4.8¶	10.9	10.7**	-0.2
	Jan. 4, 1917.....	6.0*	5.3	11.3	11.9	+0.6
	Mar. 3, ".....	4.8*	4.9	9.7	9.6	-0.1
119	Jan. 11, ".....	3.5*	6.3†	9.8	10.0†	+0.2
	Mar. 12, ".....	3.3*	8.8*	12.1	12.0†	-0.1
211	Oct. 16, ".....	7.5*	4.0	11.5	11.9	+0.4
214	Nov. 20, 1916.....	4.7*	7.8	12.5	12.7	+0.2
	Jan. 18, 1917.....	5.4†	6.1	11.5	13.2	+1.7
	Mar. 16, ".....	4.9*	6.4	11.3	11.4	+0.1
	Oct. 11, ".....	5.4*	5.3	10.7	10.8	+0.1
	Nov. 16, ".....	5.1*	5.1	10.2	9.9*	-0.3

* One determination only.

† See protocol for this animal.

‡ Check 1, 10.2; check 2, 9.3; check 3, 10.5; check 4, 8.8. All four used in calculating average.

§ Check 1, 10.5; check 2, 9.4; check 3, 10.5; all three used in calculating average.

|| First two determinations discarded on account of lack of agreement. This figure obtained in a single subsequent determination.

¶ Check 1, 4.9; check 2, 4.3; check 3, 4.8; checks 1 and 3 used in calculating average.

** Check 1, 11.6; check 2, 10.5; check 3, 10.9; check 4, 10.6; checks 2, 3, and 4 used in calculating average.

Greenwald extracts the acid-soluble phosphorus from serum with a mixture of picric and acetic acids and he has shown that none of the lipoid phosphorus is extracted from his material by this reagent.²⁶ In a later article he considers the question whether

²⁶ Greenwald, *J. Biol. Chem.*, 1913, xiv, 369.

the phosphorus extracted by the picric-acetic acid is all strictly inorganic or whether there is mixed with it a small amount of soluble organic phosphorus.¹¹

He divides his samples of serum into four portions, A, B, C, and D. In A, B, and C the protein and phosphatide are precipitated, and the acid-soluble phosphorus is extracted with picric-acetic acid mixture; in D the inorganic phosphorus is precipitated directly by magnesia mixture and subsequently determined. The picric-acetic acid extract from A is ashed with acid mixture and its total phosphorus determined; in the extracts from B and C the inorganic phosphorus is precipitated (by two different methods) and determined. The results from B, C, and D agree fairly closely with each other on the average, and are all about 0.3 mg. per 100 gm. of serum lower than those from A. Greenwald considers that in A he gets the total acid-soluble phosphorus; and in B, C, and D, the inorganic phosphorus; and he concludes that serum contains a small amount of acid-soluble phosphorus which is not strictly inorganic. To us it seems that his results might equally well be interpreted by supposing that the methods of analysis adopted in the cases of B, C, and D all give results which are a little low. It is to be noted that in all three of these analyses, and particularly in B and C, the phosphorus (small in amount) is precipitated from a large volume of fluid; this is presumably not the case in A; and it seems not unlikely that this circumstance alone might account for the lower results in B, C, and D.

However this may be, it is evident that the method used in D does not get all of the acid-soluble phosphorus from serum, the amount not accounted for being about 0.3 mg. per 100 gm. of serum. The method used in D is essentially the same as our routine method for inorganic phosphate; therefore, in order to calculate the total acid-soluble phosphorus in our samples of plasma from our routine figures for inorganic phosphate, one would have to add 0.3 mg. per 100 gm. to each of our results. This would slightly more than cover the average difference between our figures for total plasma phosphorus and those for the sum of the lipoid and inorganic; the difference in question was 0.26 mg. per 100 gm. In other words, our results show that our samples of plasma contained only lipoid and acid-soluble phos-

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phorus; the difference between the sum of these quantities and the total as determined independently is only 0.04 mg. per 100 gm. of plasma. It is evident that there can be no nuclein phosphorus in either the lipoid or acid-soluble fractions, and it follows that plasma contains no nuclein phosphorus.

Greenwald has reported an experiment²⁷ which indicates that serum contains no nuclein phosphorus. He precipitates and extracts serum with acetone, reextracts the precipitate with alcohol and ether, and finally with picrohydrochloric acid mixture; he finds only a negligible quantity of phosphorus in the residue thus extracted. No figures are given, however, from which one could judge the completeness of the demonstration that nuclein phosphorus is absent, and we feel justified in publishing our results as a confirmation and extension of his conclusion.²⁸ There remains the question whether the small discrepancy between the acid-soluble phosphorus of plasma and the inorganic as determined by the direct precipitation of magnesium ammonium phosphate without previous protein precipitation is or is not merely the result of the failure of the latter method to account for all of the inorganic phosphorus. We are inclined to think that it is. It has been pointed out above that Greenwald's results bearing on this question do not seem conclusive; and we think that weight should be given to the fact that the "inorganic phosphate" of plasma as determined by the Embden method averages a little higher than that obtained by our routine method (see above under Methods). We shall not give here a detailed account of the former Method. It is only necessary to say that Embden and his collaborators were satisfied, after testing it in various

²⁷ Greenwald, *Am. J. Med. Sc.*, 1914, cxlvii, 226.

²⁸ We fear, however, that a table published by him (*J. Biol. Chem.*, 1915, xxi, 32) may cause us to be accused of simply repeating his experiments. At first sight, this table does appear to be exactly similar to our Table I, but in the text of the article (pp. 30, 31) one finds that the figures for "lipoid phosphorus" given in Greenwald's table represent the total phosphorus left in the residue from serum extracted with picric-acetic acid mixture. We do not object to this method of estimating the lipoid phosphorus of serum, but we wish to point out that Greenwald's table has no value as a demonstration that serum contains only lipoid and acid-soluble phosphorus; it is only through the previous proof of this proposition that his figures for lipoid phosphorus attain their value.

ways, that it gave correct results for the inorganic phosphate of muscle juice. Their results were lower than most of the previous figures given for the inorganic phosphorus of muscle and muscle juice. There can be no doubt that muscle juice contains considerable amounts of labile organic phosphorus compounds, and a method capable of separating the strictly inorganic phosphorus from these would be unlikely to give high results for the inorganic phosphate of blood plasma.

If our conclusions are correct, it follows that the plasma does not serve as a vehicle for the transport of nucleins or phosphorized proteins, and that these compounds must be synthesized *in situ* by the cells in which they are found. It follows also that if one knows the total phosphorus of a sample of plasma, and either the lipoid or inorganic phosphorus, one can readily calculate the amount of the missing fraction.

Precursor in the Blood Plasma of Milk Phosphorus and Milk Fat.

Most of the work directed toward discovering in the blood the precursors of the various constituents of milk has been devoted to the question of the precursor of lactose. It has been established that the glucose of the blood is the precursor of lactose; evidence for this view has been obtained by two different methods of investigation. One of these is to remove the mammary gland from the body, to perfuse it, and to study the concentration of the materials in question in the perfusion fluid before and after it has been sent through the gland, as well as in the fluid secreted by the gland. The other method is to obtain samples of blood from one of the mammary veins and from some other superficial vein, say the jugular, in the intact animal, and to compare the concentrations of the materials in question in such samples of blood.

The second method seems to us the more promising, because when using it one is working with a gland which is functioning under much more nearly normal conditions, and there are many reasons for thinking that the functioning of the mammary gland is easily disturbed.

There are, however, objections to the second method, which must be considered. One of these is that, in a single passage of

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the blood through the mammary gland, its composition would probably be so little changed that the change could not be detected by the biochemical methods which are at present at our disposal. The most effective answer to this objection lies in the results that have been obtained by the method by other investigators as well as by us. These will be reported and discussed later. Another objection is that blood obtained from the jugular vein cannot be regarded as strictly equivalent to the blood carried to the mammary gland by its artery; this objection may be answered as follows.

The blood pumped by the left ventricle of the heart into the aorta is presumably thoroughly mixed, so that all parts of the body supplied by the aorta receive identical samples of blood. It may, therefore, be presumed that the blood supplied to the tissues drained by the jugular vein is the same as that supplied to the mammary gland. The tissues drained by the jugular vein are, for the most part, muscles, and the changes brought about by muscles in the phosphorus or sugar content of blood passing through them would be negligibly small in comparison to that brought about by the mammary gland. The blood of the jugular vein would, therefore, for all practical purposes be identical with the arterial blood going to the tissues drained by that vein, and hence also with the arterial blood going to the mammary gland.

The blood which has passed through the cow's mammary gland is to a large extent carried away by the abdominal subcutaneous veins or milk veins.²⁹ These veins run forward from the udder on the abdomen immediately under the skin; in milking cows they are usually extremely large and prominent, and blood may be obtained from them by means of a trochar and cannula rather more easily than from the jugular vein.

Our experimental procedure has consisted in obtaining samples of blood from the milk veins and from the jugular veins of milking and dry cows, as nearly simultaneously as practicable, and in analyzing the blood and plasma from such samples for total, lipid, and inorganic phosphorus. Five such experiments have

²⁹ Chauveau, A., *The comparative anatomy of the domesticated animals*, New York, 2nd edition, 1905, 711, 998-999. Sisson, S., *The anatomy of the domesticated animals*, Philadelphia and London, 2nd edition, 1914, 609, 721.

been carried out on milking cows and three on dry cows. Of these eight experiments, the results of five are reported in detail in the protocols of Experiments 1 to 5.

In Experiment 1 the chemical analyses ran smoothly. The differences between checks are less than 2 per cent throughout, and the lipoid and inorganic fractions in both jugular and mammary plasma add up to very nearly the amount of the totals as determined independently. The result seems at first sight rather surprising. Apparently the only change brought about in the plasma by its passage through the mammary gland is an increase in the amount of inorganic phosphate contained in it, amounting to 0.65 mg. per 100 gm. of plasma. As there is no change in the lipoid phosphorus, the increase in inorganic shows itself as an increase in the total, so that the mammary plasma contains more phosphorus than does the jugular.

Such a condition cannot, of course, be usual in milking cows. The mammary gland of a milking cow must, on the whole, be taking phosphorus from the blood instead of discharging phosphorus into it, and Experiment 1 must represent abnormal or unusual conditions. The subject of this experiment had been greatly disturbed by the experimental procedure (see protocol), and we, therefore, carried out further experiments in which our methods of avoiding disturbance to the subjects were gradually improved. The first two of these gave results essentially similar to those of Experiment 1, and are not reported in detail here. In the first of them the cow was almost as much disturbed before the collection of the mammary blood as in Experiment 1. In the second, she was decidedly less disturbed than in Experiment 1, and there was some indication that the lipoid phosphorus in the mammary plasma was decreased. The decrease was small, however, in comparison to the increase in the inorganic phosphorus, and our checks did not agree closely enough to satisfy us that it might not have been the result of experimental error. In this experiment we compared the total phosphorus content of the jugular and mammary blood, as well as that of the plasma, and found an increase in the total phosphorus of the mammary blood closely corresponding to the increase found in the mammary plasma.

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In Experiments 2 and 3 our methods of avoiding disturbance to the subjects before and during the taking of the mammary samples were much improved. These experiments, like the three others, indicate that, at the time they were being carried out, the mammary gland was discharging inorganic phosphate into the blood stream. But they differ from them in indicating that at the same time the gland was taking up lipoid phosphorus from the blood. In Experiment 2 enough lipoid phosphorus was taken up to compensate for the discharge of inorganic phosphate into the blood; in Experiment 3 the discharge of inorganic phosphate was decidedly larger than in any of the others and the amount of lipoid phosphorus taken up was not enough to compensate for it. But the lipoid phosphorus taken up was nevertheless larger than in the case of Experiment 2—somewhere between 0.55 and 0.74 mg. per 100 gm. of plasma.

The experiments taken together indicate that in milking cows the mammary gland is continually discharging inorganic phosphate into the blood; that, when the cow is undisturbed, the gland takes lipoid phosphorus from the blood; but that this latter process is interfered with by even comparatively slight disturbance. It seems to us that these results can best be interpreted by supposing that the gland gets from the blood both the fat and phosphorus which it requires for milk secretion in the form of phosphatide—either lecithin or some related body.

Lecithin contains about one part by weight of phosphorus to eighteen of fat; other phosphatides contain a still larger amount of phosphorus in proportion to the fat. Milk, on the other hand, contains one part of phosphorus to about 50 parts of fat. It is obvious, therefore, that if the mammary gland gets from the blood enough phosphatide to supply the milk with fat, it must get with it more phosphorus than is required for the milk, and the excess must be returned to the blood. These considerations explain the back-flow of inorganic phosphate from the gland to the blood which occurred in all of the experiments.

It will be well to continue the discussion of these experiments by referring to the work of Kaufmann and Magne³⁰ who compared the sugar content of the jugular and mammary blood of milking

³⁰ Kaufmann, M., and Magne, H., *Compt. rend. Acad.*, 1906. cxliii, 779.

and dry cows. Our experience has been, in many respects, parallel to theirs.

These authors have investigated the mammary secretion of lactose by comparing the sugar content in samples of blood obtained nearly simultaneously from the mammary and jugular veins. They found that in milking cows the mammary blood always contained less sugar than the jugular. Their experiments were carried out on different cows giving different amounts of milk and at different periods after milking, etc., so that the averages of their figures are not very significant. But their results in round numbers may be indicated by saying that the jugular blood generally contained about 0.08 per cent of sugar while the mammary blood contained only 0.07 per cent; each 100 cc. of blood in passing through the gland gave up to it about 0.01 gm. of sugar. Their cows, as nearly as can be judged from their description, gave about 10 liters of milk daily; and, by using these figures and reckoning the amount of lactose in the milk at 5 per cent, a rough calculation can be made of how much blood was passing through the mammary gland per minute in their experiments.³¹ 10 liters of milk with a 5 per cent lactose content would contain 500 gm. of lactose; in other words the cows used by Kaufmann and Magne were secreting about 500 gm. of lactose daily. They were secreting, therefore, $500 \div (24 \times 60)$ or about 0.35 gm. of lactose per minute. And, if each 100 gm. of blood passing through the mammary gland yielded to it 0.01 gm. of sugar, it would require 100×35 or 3.5 liters of blood to yield 0.35 gm. of lactose; that is, according to Kaufmann and Magne's figures, about 3.5 liters of blood were passing through the udders of their cows per minute. It is realized that this estimate is very rough; no doubt the rate at which the blood passes through the mammary gland varies enormously in different cows and under different physiological conditions. But to have even a rough idea of this rate is desirable, in order to see to what extent the

³¹ As far as we know, the experimental evidence is all in favor of the view that in lactating animals, milk secretion is going on all the time. See Roehrig, A., *Virchows Arch. path. Anat.*, 1876, lxvii, 119. Foa, C., *Arch. Fisiol.*, 1912, x, 402. See also Gaines, W. L., *Am. J. Physiol.*, 1915, xxxviii, 303. Our own results and those of Kaufmann and Magne still further confirm this view.

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results of our experiments would account for the secretion of milk fat.

The cows used in Experiments 2 and 3 were giving each about 10 liters of milk daily and about 400 gm. of fat. Their glands, therefore, would take from the blood about $400 \div (24 \times 60)$ or 280 mg. of fat per minute. Supposing that 3 liters of blood were passing through the gland per minute, this would mean that from each 100 cc. of blood passing through $280 \div 30$ or 9 mg. of fat in the form of phosphatide would be taken. As the proportion of fat to phosphorus in phosphatide is about 18:1, this would mean that $9 \div 18$ or 0.5 mg. of lipid phosphorus would be taken from each 100 cc. of blood passing through the gland. This figure corresponds satisfactorily with those which have been actually found. While we realize very fully the possible inaccuracy of many of the assumptions on which the above calculation is based, we still feel that the agreement between the expected and the experimental figures for the differences in lipid phosphorus concentration as between jugular and mammary plasma helps to confirm the view that milk fat is derived from plasma phosphatide.

It would be satisfactory if, in such experiments as those described above, determinations of total fat in the plasma could be made along with the phosphorus determinations to demonstrate that the decrease in total fat in the mammary plasma is only sufficient to cover the decrease in phosphatide. In some of the experiments fat determinations were made according to the nephelometric method described by Bloor (see above) and it was found that the plasma contained about 0.8 per cent of total fat. The expected decrease would be 0.009 per cent, only a little more than one hundredth part of the total, and we fear that none of the fat methods with which we are familiar could demonstrate such small proportional differences.

We did, however, determine the total fat in the jugular and mammary plasma in Experiment 1. The results indicated, as far as they went, that the jugular and mammary plasma contained the same amounts of fat, but the differences between the checks were very much larger than the difference that might be expected between the phosphorus contents of the two samples of plasma as the result of the taking up of fat by the mammary gland.

We endeavored to control our experiments as Kaufmann and Magne did theirs by comparing samples of jugular and mammary blood from cows that were not milking. The results are given in the protocols, Experiments 4 and 5. Considerably to our surprise, these cows also were apparently discharging inorganic phosphate from the mammary gland into the blood, and at about the same rate, as far as could be judged from the figures, as the milking cows. Neither cow appeared to be taking up lipoid phosphorus from the blood into the gland. Cow 106, Experiment 4, had been dry for only a few days, and still had a good deal of milk in her udder; Cow 115, Experiment 5, had never had a calf, and was probably not pregnant at the time the experiment was carried out.

These results made us think, at first, that our earlier results on the milking cows might have had nothing to do with milk secretion; and they caused us to look very carefully into the technique of our experiments and into the physiology of the mammary gland in "dry" cows.

As was said above, the milk vein in a milking cow is very large and close under the skin. When the animal is standing, it lies considerably lower than the jugular vein; and partly for this reason, partly, perhaps, because the blood flow through the active gland is very free, one can collect blood sufficiently rapidly from it through quite a small cannula. We found that in order to collect blood from the mammary vein, it was not necessary to fasten the cow in any special way at all. She was simply allowed to stand with her head in the stanchion as she does at all times when she is standing in her stall. One of the assistants held his arm in front of her hind legs while the mammary cannula was being inserted to avoid any possibility of effective kicking; but, in Experiments 2 and 3, in which the reduction of lipoid phosphorus in the mammary plasma was demonstrated, this precaution turned out to be unnecessary, for the cows made no attempt to kick, and, indeed, seemed hardly to notice the insertion of the trochar and cannula at all.

To collect blood from the jugular vein is not so easy. It is necessary, in the first place, to hold the cow's head so that her neck shall be more or less extended, and this procedure always causes some uneasiness. As the vein lies deeper under the skin

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than the milk vein, a larger cannula is necessary, and it is generally not quite so easy to insert it. But the process is not a complicated one. Only a few minutes elapsed in Experiments 2 and 3 between the end of the collection of the mammary blood and the beginning of the collection of the jugular blood.

The result of our first three²² experiments indicated that it takes only a small disturbance to interfere with the taking up of phosphorus from the blood by the mammary gland. It was thought advisable, therefore, to avoid disturbance as far as possible before and during the collection of the mammary sample, and for this reason the whole procedure necessary for the collection of the jugular sample was left, in Experiments 2 and 3, until after the mammary sample had been collected. It was realized that this course introduced the objection that the cow was somewhat disturbed between the collections of the two samples of blood that were to be compared with each other, but it was not thought that the disturbance would be sufficient to alter materially the phosphorus content either of the blood as a whole, or of the blood coming from the jugular vein. Further, we hoped to control this point completely by our experiments on the dry cows. But our experiments on the dry cows do not furnish a complete control in regard to this point. As far as the increase in inorganic phosphate in the mammary plasma is concerned it occurs in the dry cows as well as in the milking ones, and to a surprisingly similar extent. The decrease in the lipid phosphorus of the mammary plasma does not occur in the dry cows. The experiments on the dry cows were carried out later than Experiments 2 and 3, and we tried to make them similar to these in every respect, so that, as far as the lipid phosphorus is concerned, they do control Experiments 2 and 3 to a certain extent. But they do not do so completely, because the milk veins in dry cows are smaller than in milking cows—in heifers that have never had calves they are very much smaller. For this reason it is more difficult to insert a trochar and cannula in the milk vein of a dry cow, and the animals used in Experiments 4 and 5 showed more sign of disturbance when the cannula was in-

²² Between Experiments 1 and 2, two others were carried out which had the same results as Experiment 1. It has not been thought worth while to report them in detail.

served in their milk veins than did the animals used in Experiments 2 and 3. Both the cows used in Experiments 4 and 5 made attempts to kick. It was thought desirable, therefore, to control the experiments in another way.

The method chosen was to obtain two samples of jugular blood, one with as little disturbance as possible to the subject, and the other after the same amount of disturbance as occurred in Experiments 2 and 3. For this purpose a cow was chosen which had been dry for some time. The details of the experimental procedure and the results are given in the protocols under Experiment 6. It will be noted that there is no significant difference between the two samples of blood and plasma with respect either to lipoid or inorganic phosphorus; and the experiment may, therefore, be taken to indicate that the differences in the phosphorus content of jugular and mammary blood and plasma observed by us in Experiments 1 to 5 were not due to any change in the whole body of blood brought about by the disturbance of the subject which occurred between the collections of the mammary and of the jugular samples.

We have pointed out above that the tissues drained by the jugular vein are for the most part muscles, and that the phosphorus metabolism of muscle would be too small to affect the results of such experiments as we have been discussing. But we feel that it is worth while to consider the phosphorus metabolism of muscle more fully at this point, both on account of its bearing on the results of these experiments, and on account of its own intrinsic importance.

It has been shown by a number of workers that muscular exercise in intact animals and human beings causes an increase in the phosphate excreted with the urine,³³ and that the activity of striated muscle results in an increase in the inorganic phosphate contained within it.³⁴ These results may be taken to show that in active muscles inorganic phosphate is produced from some

³³ Flint, A., Jr., *N. Y. Med. J.*, 1871, xiii, 609. Klug, F., and Olsavszky, V., *Arch. ges. Physiol.*, 1893, liv, 21. For further references on this subject see Forbes, E. B., and Keith, M. H., A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull.* 5, 1914, 464 ff.

³⁴ Macleod, J. J. R., *Z. physiol. Chem.*, 1899, xxviii, 535.

organic phosphorus compound, and that the inorganic phosphate so formed tends to escape to the blood and to be excreted in the urine. But Macleod has shown that the muscles of animals which have been put through a period of vigorous exercise contain more total phosphorus than do unexercised muscles.³⁴ As the exercised muscles contain more total phosphorus in spite of the fact that, during exercise, muscles tend to give up inorganic phosphate to the blood, they must have taken up organic phosphorus from the blood. And, as the blood plasma contains only lipoid and inorganic phosphorus, the organic phosphorus must have been taken up in the form of phosphatide.

It would seem, therefore, that the phosphorus and fat metabolism of the mammary gland and of striated muscle are roughly parallel. Both tissues receive their fat and phosphorus from the blood in the form of phosphatide; both convert the phosphatide into fat and inorganic phosphate; they use the fat, in the one case, to supply the fat of milk, in the other, to burn and supply energy; and they return the inorganic phosphate largely or wholly to the blood. It is impossible, therefore, to explain the differences which have been observed by us in the lipoid and inorganic phosphorus content of jugular and mammary blood as the result of phosphorus metabolism carried on in the muscles drained by the jugular vein. Muscles take lipoid phosphorus from the blood passing through them and discharge inorganic phosphorus into it; and the results of our experiments show, as was to have been expected, that these processes go on much more rapidly in the case of the mammary gland than in the case of the muscles.

It is justifiable to conclude, then, that the differences in the phosphorus content of jugular and mammary plasma observed by us are the result of the metabolic activity of the mammary gland. Two aspects of the results remain to be discussed; namely, the fact that the taking up of lipoid phosphorus by the gland was never more than enough to balance its discharge of inorganic phosphorus, and the fact that such large amounts of inorganic phosphorus were discharged into the blood by the glands of the non-milking cows.

We believe that the first of these circumstances is to be explained partly by the supposition that even in our most successful experiments the subjects were enough disturbed by the procedure

to interfere to some extent with the taking up of phosphatide by the mammary gland. But we believe also that in most of the experiments the procedure caused a more than normally rapid discharge of inorganic phosphate from the gland to the blood. In all except Experiment 2 the mammary gland was more or less physically agitated. It seems not improbable *a priori* that physical agitation of the mammary gland would accelerate the discharge of phosphate from its cells to the blood, and the experiments furnish evidence indicating that this is the case.

The least difference in inorganic phosphate content of jugular and mammary blood that we obtained was in the case of Cow 211, Experiment 2. In this case the subject's udder was not disturbed at all either by milking or by any attempt on her part to kick. The greatest difference was obtained in the case of Cow 17, Experiment 3, where the calf was allowed to suck before and during the collection of the mammary blood sample, and spent much of the time when it was supposed to be sucking in butting the udder with its nose. In the other experiments, where the results were intermediate, the udder was physically agitated, though decidedly less than in Experiment 3, sometimes by having the cow milked by an assistant during the collection of the mammary sample, sometimes by her own attempts to kick.

We believe that the discharge of inorganic phosphate from the mammary gland cells to the blood in the non-milking cows is to be explained by supposing that these cells remain to some extent active for a long time after a cow has become dry, and become active in heifers before they have become pregnant for the first time. We should feel some timidity about basing this statement on the results of our blood analyses alone, but they are supported by so much independent evidence that there can be no doubt of their truth. Several cc. of milk were readily obtained from the udder of each of the dry cows used in the experiments described above, and from another cow, selected for examination because she had been dry for some time. These cows had not been milked for 6 days, 25 days, and nearly 4 months respectively. A few cc. of milk were also obtained from the udder of Cow 115, a heifer about 3 years old which had never had a calf and was probably not pregnant, though she had been bred unsuccessfully a number of times. As Cow 115 might possibly have been pregnant from

some of the later services, another heifer was selected for examination. This animal was 15 months old and had never been bred at all. A few drops of milk were obtained without much difficulty from her udder. Hill³⁶ has recently reported the case of a virgin kid, which yielded 120 cc. of milk when it was about 4 months old. It was milked at intervals for the next 3 months, 1,900 cc. being obtained altogether. The milk was analyzed and found to be normal in every way. A somewhat similar case has been reported by Pfaundler,³⁶ and Schein³⁷ has elaborated a theory of continuous milk secretion.

The difference between the concentrations of inorganic phosphate in the jugular and mammary plasma of non-milking cows is about the same as in the milking cows. We believe that this circumstance is connected with the fact that the blood flows very much less rapidly through the glands of non-milking animals. We have had occasion to make a more or less careful examination of the milk veins of mature dry and milking cows, and of young farrow heifers. In the milking cows this structure is very large, and, in the heifers, quite small; it probably carries not more than one-tenth the amount of blood in the latter case, and perhaps even much less than this. In the dry mature cows that we have examined, the milk vein has perhaps nearly the same capacity as in the milking ones, but it gives the impression of carrying very much less blood. It feels less distended, and blood flows decidedly slower from a cannula inserted in it. It seems not improbable that the activity of the mammary gland cells is dependent on the amount of blood flowing through the gland as much as on any other single factor.³⁸

Many of the features of fat and phosphorus secretion by the mammary gland, as indicated by our results, are closely paralleled by what is already known regarding the secretion of carbohydrate. It has been observed for instance by several investigators that animals in the early stages of lactation or just before parturition are likely to exhibit lactosuria. Thus von Noorden³⁹

³⁶ Hill, R. L., *J. Biol. Chem.*, 1918, xxxiii, 391.

³⁶ Pfaundler, M., *Z. Kinderh.*, 1912, iii, 191.

³⁷ Schein, M., *Wien. klin. Woch.*, 1910, xxiii, 1337.

³⁸ The same conclusion, though from very different evidence, has been reached by Roehrig.⁴¹

³⁹ von Noorden, H., *Arch. physiol.*, 1893, 385.

found that when glucose was given *per os* to a newly lactating goat, lactose appeared in the urine. Porcher has repeated this experiment on a dog and obtained similar results.⁴⁰ And Kaufmann and Magne³⁰ found that one of the cows experimented on by them exhibited lactosuria 24 hours before she calved and for 3 days thereafter. They obtained blood samples from the jugular and mammary veins of this cow 24 hours before she calved and found that the mammary blood contained considerably less sugar than the jugular, though at this time the cow was presumably giving no milk. They are extremely brief in giving their methods of analysis and their experimental results; but, as far as can be judged from what they say, the decrease in the figure obtained by them for the sugar content of mammary blood would be, at least in part, accounted for by supposing that some of the glucose was taken out of the blood by the mammary gland and that lactose was substituted for it.

There is thus evidence that the lactose which the mammary gland cells manufacture from glucose may, under certain circumstances, be partly returned to the blood, just as is a part of the phosphate which they manufacture from phosphatide; and also that before parturition, when the gland is not actively secreting milk, its cells may, nevertheless, be taking up glucose from the blood, converting it to lactose, and returning this to the blood, just as they may be taking up phosphatide, and returning the phosphate, and presumably the fat also, to the blood.

In a number of experiments on the comparison of jugular and mammary blood, it was found that the jugular blood contained a greater volume of corpuscles than the mammary (see protocols). The differences varied greatly, but were always in favor of the jugular blood, if they occurred at all. In Experiment 3 on Cow 17 the jugular blood contained 31.4 volumes per cent of corpuscles, whereas the mammary contained only 28.2 volumes per cent, so that the corpuscles in the mammary blood were decreased by more than 10 per cent of their volume in the jugular blood. It is unlikely that any osmotic changes in the plasma brought about by the taking up of water, salts, or sugar by the mammary gland could produce so large a shrinkage in the volume of the corpuscles; and it is probable, therefore, that the difference is to be

⁴⁰ Porcher, C., *Arch. internat. physiol.*, 1909, viii, 356 ff.

explained as a difference in the number of corpuscles in the two samples of blood. An examination of the figures for total phosphorus in blood and plasma in this experiment supports this view. In the case of the whole blood, the jugular sample contains more total phosphorus than the mammary, while these relations are reversed in the case of the two samples of plasma. As the corpuscles contain considerably more phosphorus per unit of volume than the plasma, this circumstance is very well explained by supposing that in this experiment the jugular blood contained a larger number of corpuscles than the mammary, and that the phosphorus contained in these extra corpuscles was more than enough to offset the lesser phosphorus content of the jugular plasma.

It would seem, therefore, that, under the conditions of our experiments the jugular blood is likely to contain a rather variably larger number of corpuscles than the mammary. If this phenomenon showed more regularity, it might conceivably be taken to mean that corpuscles had a tendency to become engaged and held in the capillaries of the mammary gland, and there broken up, while the products of their decomposition are used for milk secretion. But the greatest difference between jugular and mammary corpuscles was in the case of Cow 115, which had never had a calf, and in which there was no sign of the taking up of lipoid phosphorus by the mammary gland; while in the case of Cow 211, in which secretion was going on actively, the difference was small. It seems to us more likely, therefore, that the phenomenon is some unforeseen effect of the experimental procedure; possibly the movements of the head, which always occur as the result of the collection of the jugular sample, result in the washing out from the capillaries of the muscles drained by the jugular vein of a varying small amount of corpuscles which have collected there during the preceding period of greater quiescence.

It might be expected that, if plasma phosphatide is the precursor of milk fat, there would be some tendency to parallelism between the concentration of phosphatide in a cow's plasma at any given time and the percentage of fat in the milk secreted at the same time. With this idea in mind we have compared the phosphatide concentrations in a number of our samples of plasma with the fat in the milk yielded by the subjects of the ex-

periments at about the time the blood samples were obtained. The results have shown it to be a rough general rule that cows with a high percentage of plasma phosphatide are likely to yield milk rich in fat, but there are many individual exceptions. The most that can be said is that, while the phosphatide concentration in the plasma is probably one of the factors which influence the fat content of milk, there are other factors which often mask its influence.

The literature on the immediate precursor of milk fat is not large. The evidence which bears most directly on the question is contained in the experiments of Foà.⁴¹ This author perfused the isolated mammary glands of sheep with various fluids, and showed quite conclusively that milk secretion may still go on in the isolated gland. His results indicate also that milk fat may be derived from triglycerides in the blood.

He found that when he perfused Ringer's solution in which olive oil or triolein was emulsified, through the vessels of the gland, he obtained a fluid from the duct which contained fat. That the olive oil and triolein emulsions had not simply filtered through the gland cells into the ducts is shown by the facts that the fat in the fluid obtained from the duct was in the form of globules with the microscopic appearance of those normally seen in milk, and that it had a lower iodine number than the olive oil and triolein mixed with the perfusion fluid. That the fat in the fluid issuing from the duct was not simply fat which had been present in the gland cells before the beginning of the experiment and had been washed out into the ducts by the perfusion fluid is indicated by the fact that when Ringer's solution without fat was perfused through the vessels, there issued from the duct a fluid which contained no fat.

We think that the olive oil and triolein may have entered the gland cells from the perfusion fluid, and that they may have been partially changed within the cells to fats with lower iodine numbers; but we do not think that this proves that triglycerides enter the gland cells from the blood under normal conditions. In the three experiments of Foà which bear on the point the gland became edematous quite early, and in all of them the collection of the fluid issuing from the duct, which was afterward analyzed, was continued after the edema had been noted. What-

⁴¹ Foà, *Arch. Fisiol.*, 1912, x, 402.

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ever theory one may hold regarding the nature of cell surfaces, it seems reasonable to suppose that a perfusion fluid, which produced edema in the mammary gland in between 1 and 2 hours, would alter the surfaces of the cells directly presented to it before the edema became perceptible. Alterations in cell surfaces brought about by abnormal influences are almost always in the direction of increased permeability.

The cell surfaces of the glands used by Foà were, therefore, probably abnormally permeable, and the fact that triglycerides passed through them would not prove that they could pass through under normal conditions. But it is not necessary to suppose that an alteration in the surfaces of the mammary cells would immediately suspend all their activities. Urano⁴² has shown that striated muscle cells may still contract long after the permeability of their surfaces has been abnormally increased, and one of us⁴³ has shown that a similar retention of function may occur in clam's muscle after it has been for 40 hours in pure cane sugar solution and after most of the salts that are normally present have diffused out and become substituted by sugar. It would not be surprising, therefore, if it should turn out that the mammary cells still retained the power of acting on combinations of glycerol and fatty acids after their surfaces had been so altered as to permit the passage of materials to which they are normally impermeable.

We think that our results indicate, though they cannot be said to prove that milk fat is not normally derived from the triglycerides of the blood. In two of our experiments the taking up of phosphatide from the blood by the gland cells is directly demonstrated, and the rate at which this process occurs is, as far as can be judged from the data at hand, sufficient to account for the daily production of milk fat on the supposition that it all comes from the phosphatide of the blood. In all of the experiments there is demonstrated a considerable back-flow of inorganic phosphate from the gland to the blood, which is easy to understand on the supposition that it represents the excess phosphate brought into the gland cells with the phosphatide necessary for milk fat secretion, but is left as a very puzzling fact, if we must sup-

⁴² Urano, F., *Z. Biol.*, 1908, 1, 212. See also Fahr, G., *ibid.*, 1909, lii, 72.

⁴³ Meigs, E. B., *J. Biol. Chem.*, 1915, xxii, 496-497.

pose that milk fat is derived both from phosphatide and from triglycerides which contain no phosphorus.

Finally it seems to us an improbable supposition that the mammary gland derives its fat both from phosphatides and from triglycerides in the blood. It is our belief that phosphatide furnishes a form in which fatty acids may readily pass through cell surfaces, and we are inclined to think that the very rapid excretion of fat accomplished by the mammary gland is made possible by this circumstance.

Jordan and a number of coworkers⁴⁴ have shown that reducing the amount of phosphorus in a cow's rations sometimes reduces not only the percentage but also the absolute amount of the fat secreted with the milk. This result does not always occur, but in some cases it is quite marked and persists for some time. It is not to be expected that this result would occur with complete regularity. It has been shown repeatedly⁴⁵ that a milking cow is capable of taking large amounts of calcium and phosphorus from her bones to supply any deficiency in these elements that may exist in her rations. It is quite possible, therefore, that, unless a cow's phosphorus stores were already more or less depleted, a deficiency of phosphorus in the diet would not show itself in any disturbance of fat metabolism. But that a deficiency in the phosphorus intake results even occasionally in a decrease in the excretion of milk fat is fairly strong confirmatory evidence for the view that the phosphatide of the blood plasma is the precursor of this product.

The following is a résumé of the conclusions drawn from our experimental results. The fat and phosphorus which are excreted with milk come from some phosphatide body or bodies in the blood. This material is converted in the mammary gland cells to inorganic phosphate and neutral fat; it yields two and a half or more parts of phosphorus to forty-five of fat; and, as milk contains only about one part of phosphorus to forty-five of fat, some

⁴⁴ Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268.

⁴⁵ Hart, E. B., McCollum, E. V., and Humphrey, G. C., *Am. J. Physiol.*, 1909, xxiv, 100; Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Sta., Bull. 295*, 1916; *ibid.*, *Bull. 308*, 1917.

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60 per cent of the phosphorus which enters the gland cells with the phosphatide precursor of milk fat must be returned to the blood as inorganic phosphate. This back-flow of inorganic phosphate from the mammary gland to the blood is not necessarily synchronous with the taking up of phosphatide by the gland. The gland cells are at all times loaded with excess phosphate, and the escape of this excess to the blood may continue for some time after the taking up of phosphatide has ceased. The escape of excess phosphatide to the blood is probably accelerated by any physical manipulation or agitation of the mammary gland. The taking up of phosphatide by a cow's gland is stopped if she is subjected to an even slight psychological disturbance.

The mammary gland cells remain to some extent active after a cow has ceased to be milked, and they become active in heifers even before they have become pregnant for the first time.

Concentrations of Calcium and Phosphorus in the Blood and Their Relation to Milk Secretion.

Calcium and phosphorus are present in rather small concentration in the blood, but nevertheless participate actively in milk secretion. In the case of a cow giving 20 liters of milk per day, and having about 25 liters of blood in her body altogether, the total amount of each of these constituents contained in the whole body of blood at any time is much less than that excreted with the daily yield of milk. We must think, therefore, of these two elements as being rapidly taken out of the blood by the mammary gland and equally rapidly poured into it from other sources; the maintenance of the concentration of each of them in the blood will depend on the equalization of these two processes.

It is obvious that milk secretion must, to some extent, depend on the maintenance of a certain amount of each of these materials in the blood. If either of them should be exhausted in the blood, the secretion of milk would probably cease.

Further, the calcium and phosphorus present in the blood must come ultimately from the food, and their concentration in the blood must therefore, depend, either immediately or remotely on the amount of them supplied with the food. A knowledge of how the concentrations of these materials behave in the blood under

various circumstances can hardly fail to throw light on the various problems connected with their metabolism.

But we must strongly emphasize the point that knowledge gained from such a study alone is necessarily very incomplete. Suppose, for the sake of argument, that it is found that the concentration of some particular constituent, say phosphatide, in the blood is highly variable; and that it is desired to determine the factors on which this variability depends. The most cursory consideration will show that these factors are numerous and not easily controlled. Steps taken to alter any one of them may have unexpected effects on several of the others, and any of them may be altered during the course of an experiment by external or internal influences which it is impossible to keep under control. But in spite of these difficulties it has seemed to us worth while to attempt this line of investigation, and we think that the results already attained have at least a certain value as indications of the problems which ought in the future to be attacked.

Calcium of Blood.

The literature on the variations which occur in the concentration of calcium in the blood under normal conditions is not very large, and can be profitably considered in connection with our own results.

It is universally agreed, as far as we know, that the calcium of mammalian blood is contained in the plasma, and to a negligibly small extent, if at all, in the corpuscles. Our own experience falls in with this view; we think it justifiable to regard it as established. In our discussion we shall consider only the concentration of calcium in the plasma or serum, omitting any work which bears exclusively on the calcium of whole blood. We shall give our figures always in terms of elemental Ca; where the figures in the original articles were given as CaO, we shall calculate them back to Ca for the purpose of our discussion.

Halverson, Mohler, and Bergeim⁴⁶ have studied the calcium content of the serum in normal human beings and in patients suffering from various diseases. They worked also to some extent on plasma and found that

⁴⁶ Halverson, J. O., Mohler, H. K., and Bergeim, O., *J. Am. Med. Assn.*, 1917, lxxviii, 1309; *J. Biol. Chem.*, 1917, xxxii, 171.

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there was no appreciable difference in the calcium content of plasma and serum. In normal human beings they found that the calcium ranged between 9 and 11 mg. per 100 cc. of plasma; and, in their whole series of tuberculous patients, it ranged only from 8.4 to 11 mg. Their experience indicates that the calcium content of the serum is little affected by calcium given with the food. It was not in any case increased above normal figures by a high calcium diet.

Somewhat contradictory of this last result are some experiments on calcium feeding reported by Boggs,⁴⁷ who fed calcium lactate to dogs, and found as a result that the calcium of the blood increased from 6.3 mg. per 100 cc. to 8.6 mg., an increase of 36 per cent. It is very unlikely that feeding calcium lactate would produce any marked change in the volume of the corpuscles, and these results, therefore, seem clearly to indicate a change in the concentration of plasma calcium as the result of calcium feeding. Boggs determined the calcium in the blood and plasma of dogs kept for some time on a diet of bread and meat, and found that under these conditions it varied little. His figure for the normal calcium content of the plasma is 10 mg. per 100 cc.

Lamers⁴⁸ has made a more or less extended study of the concentration of calcium in the plasma of pregnant women and of women in labor as compared with that of non-pregnant women of child bearing age. Fourteen non-pregnant women had an average concentration of 10.8 mg. of calcium per 100 gm. of plasma; the figures ranged from 9.9 to 12.1 mg. For thirteen pregnant women the average was 11.6 mg.; the lowest figure was 10.6 mg., and the highest 12.8 mg. For eight women in labor the average was 12.8, the lowest figure 11.5 mg., and the highest 14.1 mg.

Determinations of the calcium content of horse serum have recently been made by Rona and Takahashi.⁴⁹ They find that under ordinary circumstances horse serum contains about 12 mg. of calcium per 100 gm.

We have determined the calcium content of plasma in pregnant and lactating mature cows and in heifers of various ages from birth onward. We have also carried out experiments to determine the effects of feeding a soluble calcium salt (calcium chloride) on the calcium concentration of the plasma. Our results for mature cows are given in Table II. They indicate that in cows the calcium content of the plasma is quite constant and not affected by either pregnancy or lactation.

The results of calcium determinations in the plasma of heifers at various ages are given under Experiments 11, 12, 13, 14, and 15. For the most part these results lend further support to the view that the plasma calcium of cattle is quite constant, and they

⁴⁸ Boggs, T. R., *Bull. Johns Hopkins Hosp.*, 1908, xix, 201.

⁴⁹ Lamers, A. J. M., *Z. Geburtsh. u. Gynäk.*, 1912, lxxi, 393.

make it appear that in cattle there is no rise in the concentration of this element as the result of pregnancy. The figures for the farrow heifers average higher than those for the pregnant cows. We do not wish to imply, however, that we think Lamers' results are purely a matter of chance or caused by experimental error. There are at least two important differences between his experiments and ours. One of these is that the subjects of his experiments were probably on an unrestricted diet, while the rations of cows are always restricted; the other, that his pregnant and non-pregnant women were probably much more nearly of the same average age than were our heifers and pregnant mature cows.

TABLE II.

Calcium in Plasma of Pregnant and Lactating Cows. Mg. per 100 Gm. of Plasma.

Pregnant, not lactating.			Pregnant and lactating.				Lactating, not pregnant.		
No. of cow.	Plasma calcium.	Days pregnant.	No. of cow.	Plasma calcium.	Days pregnant.	Days lactating.	No. of cow.	Plasma calcium.	Days lactating.
	mg.			mg.				mg.	
9	9.6	270	21	9.1	36	152	9	8.9	26
19	10.2	236	35	9.9	45	91	19	10.2	21
27	8.9*	234	54	9.7	176	254	106	9.8	220
48	9.7	251					107	10.2	40
Average. 9.6			9.6				9.8		

* This figure represents the average of the following four determinations. Check 1, 9.5; check 2, 8.9; check 3, 8.7; check 4, 8.7. All other figures for calcium in this table are based on duplicates which checked within 5 per cent.

Our experiments indicate that the plasma calcium tends to become lower with advancing age, at least up to 6 months. In three of them (Experiments 11, 12, and 13) we secured blood samples when the heifers were only 1 or 2 days old, and in two of these cases, the plasma calcium was decidedly higher than in any of the other samples that we have examined—13.4 and 13.7 mg. per 100 gm. of plasma respectively. The heifers which gave these high figures were afterward fed calcium chloride and sodium phosphate respectively; but, at the time the samples were taken, they had had nothing to eat except the small amount of colostrum which a new born heifer is likely to take from her dam.

Heifer 81, Experiment 12, was fed calcium chloride in addition to the usual rations throughout the period of the experiment (see protocols). Her plasma calcium was a little higher on the average than that of the other three heifers, but the difference is not great.

Under Experiment 7 are given the results of a short time calcium feeding experiment, more or less comparable to those of Boggs mentioned above. There was a very slight increase in the plasma calcium after the calcium feeding but the difference was within the limits of experimental error for our single determinations.

The results so far given would make it appear that in adult human beings and in cattle above the age of 2 months the plasma calcium is little if at all altered by pregnancy or lactation or by feeding rations rich in calcium. We are inclined to agree with the suggestion given by Halverson, Mohler, and Bergeim that, under ordinary conditions, the plasma calcium is kept nearly constant by means of the enormous calcium reserve stored in the bones.

But the high figures which we obtained in the case of the new born heifers call for an explanation; and we think that the key to the situation is given in an interesting article by Allers and Bondi,⁴⁹ who found that they could double the concentration of calcium in the blood of rabbits by feeding moderate doses of hydrochloric acid.

In a series of recent articles by Van Slyke and his associates⁵⁰ it has been shown that the best criterion for acidosis is the concentration of bicarbonate in the blood plasma. In acidotic conditions, the bicarbonate concentration of the plasma is uniformly depressed. Feeding acid, therefore, would tend to depress the bicarbonate concentration in the plasma, and that an inverse relation between the calcium and bicarbonate contents of the plasma is highly probable is shown by the work of Rona and Takahashi.²² These authors studied various mixtures of calcium

⁴⁹ Allers, R. A., and Bondi, S., *Biochem. Z.*, 1907, vi, 366.

⁵⁰ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289. Van Slyke, D. D., *ibid.*, 347. Cullen, G. E., *ibid.*, 369. Fitz, R., and Van Slyke, D. D., *ibid.*, 389. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *ibid.*, 401. Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *ibid.*, 405.

and bicarbonate by physicochemical methods and found that the relation between the hydrogen ion concentration and the calcium and bicarbonate contents of such mixtures was expressed by the equation, $\frac{(\text{Ca}^{++})(\text{HCO}_3^-)}{(\text{H}^+)} = \text{a constant}$. The equation means, of course, that if the hydrogen ion concentration remains constant the amount of calcium held in solution will vary inversely with the bicarbonate.

The physiological results which are at present at hand accord very satisfactorily with the view that the calcium and bicarbonate concentrations of plasma tend to vary inversely. The most striking, of course, are those of Allers and Bondi referred to above. It may well be that the bicarbonate concentration of the blood in their experiments was reduced to half by the acid feeding.

Lamers' results showing an increased plasma calcium in pregnancy are to be placed side by side with the work of Hasselbalch and Gammeltoft,⁵¹ who showed that in pregnancy in human beings there is a definite tendency for the hydrogen ion concentration of the blood at a fixed CO_2 tension to be increased. And his results on the high blood calcium of women in labor are to be considered in connection with the well known tendency of severe muscular effort to cause lactic acid to be thrown into the blood. Finally our own results, indicating a high calcium content in the plasma of very young animals, are to be taken in connection with Marriott's conclusion that the blood of infants has a higher "fixed acidity" than that of adults⁵² and that in infants the alveolar CO_2 tension is low.⁵³ We hope at a later date to compare the Ca and HCO_3 content of the same samples of plasma taken under various conditions.

Phosphorus of Blood.

The phosphorus of the blood, unlike the calcium, is contained in greater concentration in the corpuscles than in the plasma. If

⁵¹ Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 206.

⁵² Marriott, W. McK., *Arch. Int. Med.*, 1916, xvii, 847.

⁵³ Marriott, *J. Am. Med. Assn.*, 1916, lxvi, 1596.

40 Phosphorus and Calcium Metabolism

one looks in Abderhalden's tables⁴⁴ for the phosphorus content of the blood in various domesticated mammals, one finds that the corpuscle content varies widely in different animals. But one gets the impression from Abderhalden's figures that the phosphorus content of the serum is rather constant.

Greenwald,⁴⁵ however, has recently reported a series of twelve determinations of total, lipid, and "acid-soluble" phosphorus in the blood serum of several normal human beings. It appears from his figures that the phosphorus of the serum is quite variable. The total varies from 9.54 to 19.65 mg. per 100 cc. of serum; the lipid phosphorus, from 6.64 to 12.95 mg., and the "acid-soluble," from 1.97 to 6.80 mg. Very large variations occur in the "acid-soluble" phosphorus of the serum of the same individual collected at different times.

It was clear from the beginning of our work on the phosphorus content of the blood in cattle that both the corpuscle and the plasma phosphorus were highly variable, not only from individual to individual, but also in the same individual examined at different times. We have endeavored to determine some of the factors on which this variability depends.

The subject, as will be seen, is rather complicated. We have to consider separately the phosphorus of the plasma and that of the corpuscles, and each of these fractions of the blood contains two or three well marked classes of phosphorus compounds. In our work we have laid the chief emphasis on the plasma phosphorus, but in each sample of blood, we have determined the volume of the corpuscles and the total phosphorus for the whole blood as a matter of routine; from these figures, in connection with the figure for the total phosphorus of the plasma, the total phosphorus contained in the corpuscles may be calculated. In some samples of blood we have determined the lipid phosphorus of the whole blood as well as that of the plasma, and, from our figures for these samples, the lipid phosphorus of the corpuscles can be calculated. In much of our later work, the lipid and inorganic phosphorus have been directly determined in each sample of plasma.

⁴⁴ Abderhalden, *Z. physiol. Chem.*, 1898, xxv, 65.

⁴⁵ Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

We have studied particularly the changes which are produced in the phosphorus of the blood by increasing age, by changes in ration, and by pregnancy and lactation. It will be well to begin this part of our report by stating that all these changes produce effects, and that the problem is, therefore, one in which the influences of a number of factors, which may either reinforce or neutralize each other, must be disentangled⁶

Effects of Increasing Age on Blood Phosphorus.

Some idea of the changes which occur in the total phosphorus content of the corpuscles and in the lipoid and inorganic phosphorus content of the plasma as the accompaniment of increasing age may be gained by studying the figures given in Experiments 11 to 15.

For the first few days after birth the corpuscle phosphorus is very high—about twice as high as in older animals. This condition disappears rapidly; it is much diminished at the end of 2 months and hardly noticeable at the end of 4. The lipoid phosphorus in the plasma of new born calves is very low, and increases gradually up to the age of about a year. The inorganic phosphorus of the plasma is fairly high in the new born calves, but tends to increase for some time and reaches a maximum at about the age of 6 months. It is likely to fall off again at some time between this age and that of 18 months.

Effects of Change in Rations on Blood Phosphorus.

We have two experiments, Nos. 9 and 10, in which the effects of a change in rations on the phosphorus content of the blood are quite clearly shown. The subjects were two cows which were near the height of lactation. Blood samples were obtained after they had been on the comparatively high grain and hay rations ordinarily fed to the milking cows at Beltsville, Md., and again at 10 day intervals while the grain and hay rations were very much cut down—cut down, that is, to the amount usually fed to the dry cows.⁶⁶ In these experiments we followed the total phos-

⁶⁶ Most of the phosphorus in the rations fed to these cows was contained in the grain.

phorus in both blood and plasma, the volume per cent of the blood corpuscles, the lipid and inorganic phosphorus and the nitrogen in the plasma, and we calculated the total phosphorus in the corpuscles. In addition we followed the milk yields.

As was to have been expected, the low feeding produced no consistent changes in the nitrogen content of the plasma. But the results in the cases of the other constituents studied are quite marked and consistent, and they seem to us significant.

In the case of Cow 39, Experiment 10, the rations were cut down to a lower point than in the other experiment and the results are more marked and consistent. We shall, therefore, consider her case first, and later discuss that of Cow 33 in connection with it.

In Cow 39 the total phosphorus in both blood and plasma, the lipid and inorganic phosphorus in the plasma, the total phosphorus in the corpuscles, and the volume per cent of the corpuscles fell off during the period of low feeding and recovered when the cow was put back on higher rations. The milk yield also fell off quite markedly during the period of low feeding, and recovered subsequently, though not to the level which it had before the low feeding was begun. Fig. 1 gives a graphic representation of the changes in lipid and inorganic phosphorus in the plasma which occurred in this experiment.

The changes in the lipid phosphorus in the plasma are decidedly less marked than those in the inorganic; they do not occur until later, and the recovery begins before the period of low feeding is finished. During the subsequent period of high feeding, the lipid phosphorus shoots up above the concentration which it had at the beginning of the experiment.

The results in the case of Cow 33 are more or less the same as those that have just been discussed, except that they are smaller and more irregular, and that there appears to be a marked discrepancy between the two experiments in regard to the changes which took place in the concentrations of lipid and inorganic phosphorus in the plasma during the first 10 days of the low feeding. The figures would make it appear that the lipid phosphorus decreased markedly in the case of Cow 33 during this period, while the inorganic phosphorus increased.

The figure for inorganic phosphorus for the plasma of this cow obtained September 27, 1916, (at the end of the preliminary period

of high feeding) is calculated by subtracting the lipid from the total phosphorus of the same sample, and we are inclined to question the figure given for lipid phosphorus. It is true that this figure is based upon two concordant determinations by the nephelometric method, but they were among the first that we made by this method; and, in the light of our later experience, we should not be surprised if, using the procedure that we then used, we got two concordant results, both of which were 10 or 20 per cent off. If the figures are correct, they indicate an unusual temporary variation in the lipid and inorganic phosphorus of the

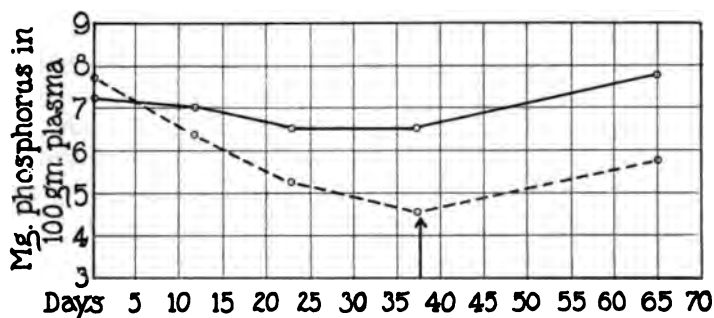


FIG. 1. Changes in the lipid and inorganic phosphorus content of the plasma which are brought about by a reduction in the grain and hay rations of a milking cow. The solid line represents the lipid phosphorus; the broken line, the inorganic phosphorus. The rations were reduced immediately after the first determinations of plasma phosphorus had been made, and were restored to the original level at the point marked by the arrow. See Experiment 10, Cow 39.

plasma. It is contrary to all our experience that feeding a reduced ration should be accompanied by an increase in the inorganic phosphate of the plasma.

In view of our present very scanty knowledge of the physico-chemical nature of secretion and of the causal connections between rates of secretions and concentrations of the precursors of gland products in the blood, a detailed discussion of the results of these experiments would be unprofitable. But the results seem to us to suggest certain views. We shall consider, chiefly in this discussion, the case of Cow 39, on account of the unfortunate doubt concerning the preliminary figures for Cow 33.

The results suggest, in the first place, that the amount of milk secreted is independent of the concentration of lipoid phosphorus in the plasma, at least within the limits of concentration encountered in this experiment. In the early part of the experiment the milk yield drops off sharply; and the plasma phosphatide, hardly at all; while, during the final high feeding period, the phosphatide shoots above the concentration that it had at the beginning and the milk yield only partially recovers toward its original level. We do not think this at all inconsistent with the view that phosphatide is the precursor of milk fat and milk phosphorus. These are only two of many milk constituents, and it is probable that the total amount of milk secreted depends on quite other factors. During the period of low feeding, there is a more or less clearly marked inverse relation between the concentration of phosphatide in the plasma and the daily milk yield. We are inclined to explain this by supposing that the phosphatide was poured into the plasma at a nearly constant rate during this period, and that its concentration in the plasma therefore varied inversely with the rate at which it was excreted in the milk. The same sort of argument applies to the rise in plasma phosphatide above its original level during the subsequent period of high feeding. Here the high feeding caused the phosphatide to be poured into the plasma at approximately the same rate as in the preliminary period of high feeding, but, as the daily milk yield was decidedly less, the concentration rose even above its original level. This part of the discussion applies with equal force to both experiments.

The effects on the milk yield of decreasing and then increasing the rations are clearly marked in both experiments, and we cannot help being impressed with the parallelism exhibited between the daily milk yield and the concentration of inorganic phosphate in the plasma. The parallelism occurs throughout the experiment on Cow 39 and throughout that on No. 33 also, except for the doubtful preliminary figures which have been already discussed. We are not ready, however, to draw the conclusion that the concentration of inorganic phosphate in the plasma has an influence on the milk yield. The reduction in milk yield and in plasma phosphate may have been parallel effects of the reduction in the grain and hay rations, which had nothing directly to

do with one another. We are at present carrying out further experiments along this line.

The results given in Experiments 11, 12, 13, and 14 indicate that feeding a soluble inorganic phosphate tends to raise the concentration of the plasma phosphate in heifers. The subjects of these experiments were four heifers, which were all fed the same basic rations from birth onward. One, however, Heifer 119, was given daily doses of disodium phosphate in addition, and another, No. 81, daily doses of calcium chloride. The other two were fed nothing in addition to the basic rations. The figures for inorganic phosphate in the plasma of Heifer 119 are decidedly higher on the average than those for either of the controls or for the calcium heifer. It is not to be expected that the difference would be very marked in such experiments as these, for the control rations contained what is generally supposed to be a quite adequate amount of phosphorus.

It is an interesting fact that the plasma of the heifer fed calcium contained less inorganic phosphate on the average than that of any of the others. That feeding calcium chloride tends to reduce the inorganic phosphate in the plasma is shown again in the results of Experiment 7. It is very well known that the feeding of soluble calcium salts tends to reduce the amount of phosphorus excreted in the urine and to increase that excreted in the feces. It seems to us the most natural explanation for these results, as well as for ours on the blood, that calcium interferes with the absorption of phosphates by precipitating them as insoluble calcium phosphate within the gut.

Effects of Pregnancy on Blood Phosphorus.

We have as yet no data for the changes in the phosphorus content of the blood which occur in the beginning of pregnancy uncomplicated by lactation. But we have figures which indicate that toward the end of pregnancy the phosphorus content of the blood and plasma is likely to be reduced. In the case of Cows 19 and 39, Experiments 8 and 10, we secured blood samples which show this tendency. Both these cows were on a constant ration. The blood samples under discussion were secured January 30, and March 9, 1917, in the case of Cow 19, and May 28 and July

24, 1917, in the case of Cow 39. All four samples were obtained after lactation had ceased. In both cases the total and inorganic phosphorus of the plasma falls off as pregnancy proceeds (Experiments 8 and 10).

In four other cases we noted a drop in the phosphorus content of the blood or plasma toward the end of pregnancy, although the phosphorus-containing portions of the rations were increased after the first blood samples had been taken. One of these cases is given under Experiment 15; the blood samples in question are those obtained March 20 and May 2, 1918. Three other cases occurred in experiments which we have not thought it worth while to report in detail. The phosphorus in the blood and plasma fell off toward the end of pregnancy in spite of increases in the phosphorus-containing portions of the rations.

We have no contrary results, and we are inclined to regard it as established that there is a tendency for the phosphorus content of the blood and plasma to fall off toward the end of pregnancy—a tendency which is largely independent of the rations, and which shows itself most constantly in the inorganic phosphate of the plasma. The point may be of considerable practical importance. It would be unprofitable to discuss it at any length in the present scanty state of our knowledge, but it seems worth while to recall the fact that, toward the end of pregnancy, phosphorus is rapidly deposited in the bones of the growing embryo.⁵⁷ Whether this alone accounts for the reduction of phosphorus in the blood, or whether it is reinforced by a decreased ability to absorb phosphorus or by an increased tendency to excrete it in the urine and feces, the hint that cows may often require more of this element than they get under ordinary methods of feeding toward the end of pregnancy would seem to be equally plain.

Effects of Lactation on Blood Phosphorus.

In order to determine the changes in the phosphorus content of the blood which accompany the transition from pregnancy to lactation, we carried out three experiments in which cows were

⁵⁷ Forbes and Keith. A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull. 5*, 1914, 110.

kept on constant or nearly constant rations through the latter part of one lactation period, the succeeding dry period, and the early part of the next lactation period; and in which the total phosphorus in the blood, and the lipid and inorganic phosphorus in the plasma were followed. The results are given under Experiments 8, 9, and 10.

The changes which occur in the phosphorus of the plasma are quite striking in these experiments, and we shall discuss them first. A graphic representation of them is given in Figs. 2, 3,

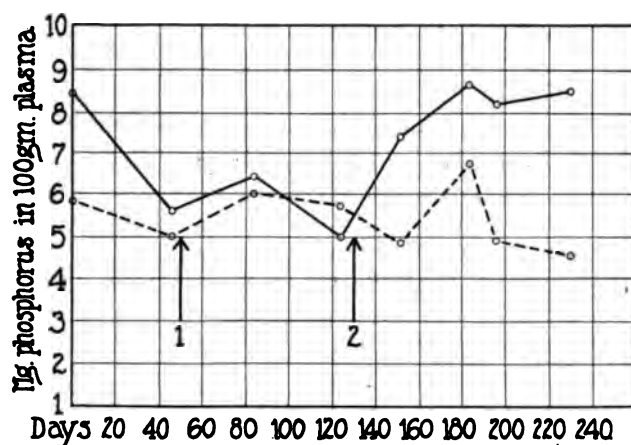


FIG. 2. Changes in the lipid and inorganic phosphorus content of the plasma which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The solid line represents the lipid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 8, Cow 19.

and 4, both lipid and inorganic phosphorus included. In all three cases the lipid phosphorus falls off quite markedly at the end of the first lactation period, remains low during the dry period, and rises again in the second lactation period. The rise in the second lactation period does not occur immediately (Figs. 3 and 4) but is marked at the end of 28 days (Fig. 2). The changes in the inorganic phosphorus are rather irregular; in two cases (Figs. 2 and 3), it tends to fall off in the early period of lactation when the lipid phosphorus is rising. It is to be noted that the absolute figures for lipid phosphorus in these three cows in their

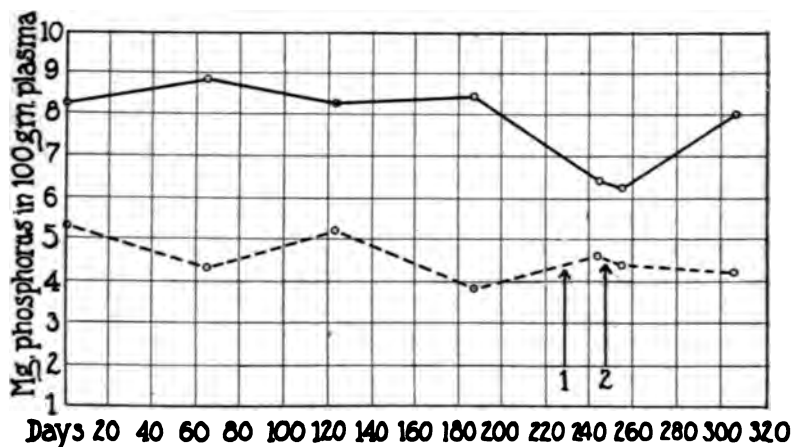


FIG. 3. Changes in the lipid and inorganic phosphorus content of the plasma, which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The solid line represents the lipid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 9, Cow 33.

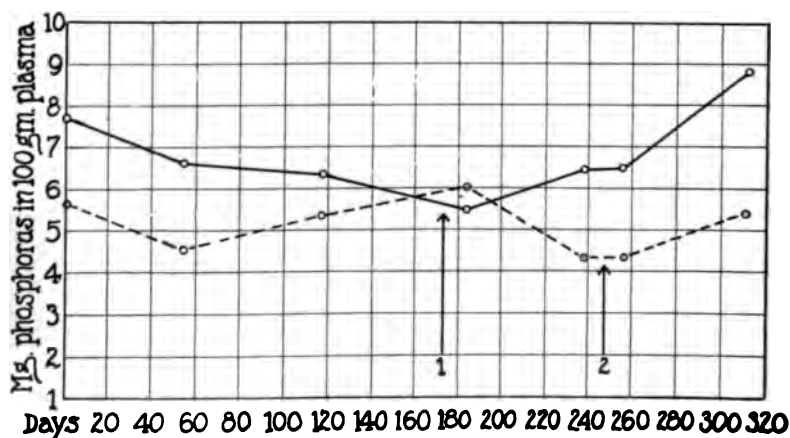


FIG. 4. Changes in the lipid and inorganic phosphorus content of the plasma, which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The unbroken line represents the lipid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 10, Cow 39.

milking periods are considerably higher than any figures that we have ever obtained in non-milking animals. They run, for the most part, between 8 and 9 mg. per 100 gm. of plasma, and we have never encountered a lipid phosphorus as high as 7 mg. per 100 gm. in any non-milking animal.

For the changes in the volume of the corpuscles, in the total phosphorus of the corpuscles, and in the nitrogen of the plasma which accompany the transition from pregnancy to lactation, the reader is referred to the figures given in the experiments. These changes do not seem to us sufficiently regular to call for any discussion.

We have a few results which bear on the lipid phosphorus of the corpuscles and on the total fat of the plasma as influenced by lactation. It has not been convenient to include these results in the protocols of the experiments, but we think they are sufficiently interesting to warrant our giving a report of them here.

In the case of Cow 19, the lipid phosphorus of the whole blood was determined on March 9, 1917, 3 days before she had her calf, and again on April 9 and June 22, 1917, after she had been milking 28 and 102 days respectively. From these figures the lipid phosphorus of the corpuscles can be calculated; it was 0.0137 per cent on March 9, 0.0139 per cent on April 9, and 0.0152 per cent on June 22. The figures indicate, as far as they go, that the phosphatide content of the corpuscles tends to rise in lactation, somewhat as does that of the plasma, though to a less extent.

Total fat was determined in the plasma of Cow 19 on January 30, 1917, 41 days before she calved, and again on May 9, after she had been milking for 58 days. The determinations were made by the nephelometric method referred to in the description of our methods. It was found that on January 30 the total plasma fat was 543 mg. per 100 gm.; on May 9, 783 mg. per 100 gm. On these dates the lipid phosphorus content of her plasma was 6.4 mg. and 8.7 mg. per 100 gm. respectively. The ratio 543: 6.4 is about 85; that between 783 and 8.7 is 90; in other words, the ratio between the fat and phosphatide contents of the plasma tends to remain relatively constant through quite wide variations in the concentrations of the two materials. We have other results which show the same thing, but we do not think it

necessary to give them in detail. Bloor⁵⁸ and others have previously reached the conclusion that the ratio between total fatty acids and "lecithin" in the corpuscles and in the whole blood tends to remain constant.

Our results on the changes in plasma phosphorus and fat, which accompany the transition from pregnancy to lactation, may be summed up by saying that in the 1st month of lactation there is a marked tendency for the fat and phosphatide to rise, that this rise occurs even in the face of a reduced ration, and that it is likely to be accompanied by a fall in the concentration of inorganic phosphate in the plasma. There can be no doubt that fat and phosphorus are taken out of the blood more rapidly by the active mammary gland than by the unborn calf at any stage of its embryonic existence; and the results given therefore indicate that some physiological change occurs near the beginning of lactation which causes fat and phosphatide to be thrown into the blood more rapidly than they are under ordinary circumstances and that this condition persists until lactation ceases. We are inclined to connect the changes which have been demonstrated with the well known tendency of good dairy cows to get thin during their lactation period and to suppose that the primary change is a mobilization of the body fat.

General Considerations in Regard to Phosphorus and Fat Metabolism.

From the literature, from the results which have been reported, and from others that are not yet ready to report in detail, we have formed some hypotheses in regard to phosphorus and fat metabolism. We think it worth while to give an outline of them, though they cannot yet be regarded as definitely established.

The recent work of Bloor⁵⁹ on fat metabolism is so well known that no extended discussion of it need be given here. His results indicate that fat received by the body from the alimentary tract is very soon largely or completely converted into phospho-

⁵⁸ Bloor, *J. Biol. Chem.*, 1916, xxiv, 456; 1916, xxv, 582.

⁵⁹ Bloor, *J. Biol. Chem.*, 1914, xix, 1; 1915, xxiii, 317; 1916, xxiv, 447; 1916, xxv, 577. Bloor, W. R., and MacPherson, D. J., *ibid.*, 1917, xxxi, 79.

tide by the red blood corpuscles. This result implies that at least some of the organs and tissues of the body can receive their supply of fat and phosphorus from the blood only in the form of phosphatide. We think that the evidence already given in this article shows that this is the case for the mammary gland and for muscles.

There is satisfactory evidence independent of Bloor's to show that the body can synthesize phosphatide from triglycerides and inorganic phosphate.⁶⁰ To us it seems probable that the body not only can, but always does supply itself with phosphatide in this manner. Our results taken altogether give us a strong impression that it is the inorganic phosphate of the plasma which is chiefly influenced by changes in ration, and the same conclusion has been reached by Greenwald.¹⁴ Such studies as have been made on the blood, therefore, indicate that it is only as inorganic phosphate that phosphorus from the digestive tract reaches the general circulation. But other considerations make it seem improbable that phosphatide introduced *per os* would escape being broken down into its components by the intestinal ferments, or that so complicated an organic compound would be received unchanged into the blood.

The facts given on pages 10 to 17 indicate that phosphorized proteins are not carried from one part of the body to another at all by the plasma, and they point to the conclusion that these compounds are always manufactured within the cells in which they are found.

We are inclined to think, then, that phosphorus from the digestive tract reaches the general circulation only in the form of inorganic phosphate, that all organic phosphorus compounds are synthesized within the body cells, and that phosphorized proteins are not transported at all by the plasma from one fixed cell in the body to another.

We think also that many of the tissues and organs of the body, of which the mammary gland and the muscles are conspicuous examples, can receive their fat and phosphorus from the blood only in the form of phosphatide.

⁶⁰ McCollum, E. V., Halpin, J. G., and Drescher, A. H., *J. Biol. Chem.*, 1912-13, xiii, 219. Fingerling, G., *Biochem. Z.*, 1912, xxxviii, 448.

SUMMARY.

1. Normal blood plasma contains no phosphorized proteins, and probably no phosphorus compounds at all except phosphatides and inorganic phosphates. The phosphorus of these two classes of compounds certainly comprises more than 97 per cent of all that exists in normal plasma.

2. The precursor in plasma of milk fat and milk phosphorus is phosphatide—either lecithin or some related body.

3. The concentration of calcium in the plasma of cows is quite constant. Small variations can be induced by varying the amount supplied with the rations, but the chief controlling factor is probably the concentration of bicarbonate in the plasma. It is probable that the concentration of calcium tends to vary inversely with that of the bicarbonate.

4. The concentrations of phosphatide and of inorganic phosphate in the plasma are highly variable. Both can be made to vary by changing the amount of phosphorus supplied with the rations, though the variations induced in this manner show themselves most markedly in the inorganic phosphate. Both undergo variations as the accompaniment of increasing age and of the later stages of pregnancy. The phosphatide of the plasma shows a marked tendency to rise during the 1st. month of lactation and to remain high until lactation has ceased. This phenomenon is largely independent of the diet, and is thought to be connected with the fact that near the beginning of lactation there is a tendency for the body fat to be released from its stores and thrown out into the blood.

In conclusion we wish to express our most sincere gratitude to Mr. T. E. Woodward, Superintendent of the Dairy Division Farm at Beltsville, Md. Our work has been carried out entirely on this farm, and has been made possible largely through Mr. Woodward's interest and cooperation.

PROTOCOLS.

In order to save space we have not published the protocols of our chemical analyses in the usual way. Our analytical methods are described above, and the quantities of material which we took

for each kind of analysis are there indicated in a general way. We feel that this gives a sufficiently accurate idea of the amount of material that we had to weigh or titrate in each case.

We made duplicate determinations in all except certain special cases, in which our results were checked in some other way. For instance, after we found that the lipoid phosphorus in a sample of plasma could be calculated within the limits of error of our determinations by subtracting the inorganic from the total, we frequently made only one determination of the lipoid phosphorus in samples in which the total and inorganic were determined independently.

We have not published our duplicate figures, however, except in certain special cases. The figures given in our tables and protocols represent, unless otherwise indicated, the average of duplicates which agreed within 5 per cent.⁶¹ Where our duplicates did not agree so closely as this, we have given the details in footnotes appended to the tables and protocols. We have published all our duplicate figures in Experiments 1 to 7. In these experiments, the differences to be demonstrated were close to our limits of error, and we feel that it is necessary to say a word about our special mode of procedure for these cases.

It has been our experience that if two analyses are carried along side by side, starting with the same quantities of the same materials and using measured and equal quantities of reagents throughout, the results are likely to agree closely with one another, even though the method may be faulty, and the two results may together diverge widely from what is known to be the correct one. For this reason, in making up our checks, we have usually taken rather different quantities of material. But, in comparing our jugular and mammary samples, we have taken equal quantities of each kind of blood or plasma, our method being as follows: Equal samples of jugular and mammary plasma or blood are weighed out, and the two are carried along as nearly as possible exactly parallel. This set of analyses is checked by another set, consisting again of a sample of jugular plasma and one of mammary plasma, the two being equal to each other, but

⁶¹ *I.e.*, the difference obtained by subtracting one figure from the other was 5 per cent or less of the larger.

both different in amount from the first set. Throughout the experiments, we have endeavored to carry through each set consisting of one jugular and one mammary sample at the same time and as nearly as possible under the same conditions; in the case of the check sets, no special care was taken to make them parallel to their checks, and the amounts of material were purposely chosen different. It seems to us that if, in such experiments, the jugular and mammary samples differ from each other in phosphorus content more than they do from their own checks, it is justifiable to assume that a difference in the phosphorus content of the jugular and mammary samples has been demonstrated.

Experiment 1. Comparison of Jugular and Mammary Plasma Obtained from Cow 19.—The animal had calved normally on March 12, 1917, and, at the time of the experiment (May 9), was giving 29 pounds of milk daily with a fat content of about 5 per cent. She was milked for the last time before the experiment at 6.00 p.m. on May 8. At 8.00 a.m., May 9, her hind legs were tied together to prevent kicking, and a trochar and cannula were inserted in her jugular vein. The cannula was left stoppered in the vein, and an attempt was made to insert another trochar and cannula in the milk vein. Before this could be done, however, the cow struggled and fell down in the stall; it was then found that blood would not run from the jugular cannula, and this was removed.

A trochar and cannula were inserted in the milk vein, and the cannula was left stoppered in the vein while the cow was milked. The milk did not run freely, but about 6 pounds were obtained; 500 cc. of blood were then collected from the cannula while the cow was still being milked; the blood ran very freely. Immediately after finishing the collection of the mammary blood, a trochar and cannula were inserted in the jugular vein and 500 cc. of blood were obtained from it, the milking being continued through the whole process. About a minute elapsed between the ending of the collection of the mammary blood and the beginning of that of the jugular blood. Both samples of blood were oxalated and centrifuged, and the two samples of plasma were analyzed for total, lipid, and inorganic phosphorus, and for nitrogen.

Results in Mg. per 100 Gm. of Plasma.

Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 15.62	16.36	8.79	8.79
II 15.70	16.32	8.70	8.70
Average. 15.66	16.34	8.74	8.74
Total phosphorus increased 0.68 mg. per 100 gm. in mammary plasma.		Lipoid phosphorus equal in jugular and mammary plasma.	
Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations, as compared with the total.	
Jugular.	Mammary.	Jugular.	Mammary.
I 6.79	7.56	Lipoid..... 8.74	8.74
II 6.85	7.39	Inorganic. 6.82	7.47
Average. 6.82	7.47		
Inorganic phosphorus increased 0.65 mg. per 100 gm. in mammary plasma.		Sum..... 15.56	16.21
		Total..... 15.66	16.34
Nitrogen.			
Jugular.		Mammary.	
I 1.253		1.253	
II 1.230		1.234	
Average. 1.241		1.243	

The blood corpuscles had the same volume, 32.5 volumes per cent in both jugular and mammary blood.

Experiment 2. Comparison of Jugular and Mammary Blood and Plasma Obtained from Cow 211.—The animal had calved normally on July 25, 1917, and, at the time of the experiment (October 16), was giving 23 pounds of milk daily with a fat content of 2.9 per cent. She was milked about 4 hours before the experiment, and, during the collection of blood, her udder was not touched. All precautions were taken to have her disturbed as little as possible before and during the collection of the mammary blood. She and the other cows of the herd were left in their stalls from the time of the morning milking until the blood was collected between 8.30 and 9.00 a.m. The men who helped

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in taking the blood were quiet. No precautions were taken to keep her in place, except that one of the men placed his arm in front of her hind legs to prevent kicking. She did not resent this and she made no attempt to kick; almost no sign of pain was

Mg. per 100 Gm. of Plasma or Blood.

Plasma.			
Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 11.71	11.97	7.49	6.89
II 12.07	11.81	Lipoid phosphorus decreased 0.60 mg. per 100 gm. in mammary plasma.	
Average. 11.89	11.89		
Total phosphorus equal in jugular and mammary plasma.			
Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations as compared with total.	
Jugular.	Mammary.	Jugular.	Mammary.
I 3.95	4.31	Lipoid..... 7.49	6.89
II 4.08	4.58	Inorganic. 4.01	4.44
Average. 4.01	4.44	Sum..... 11.50	11.33
Inorganic phosphorus increased 0.43 mg. per 100 gm. in mammary plasma.		Total..... 11.89	11.89
Blood.			
Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 17.68	17.60	10.04	9.86
II 18.09	18.16	10.36	9.48
Average. 17.88	17.88		9.89
		10.20	9.74
Total phosphorus equal in jugular and mammary blood.		Lipoid phosphorus decreased 0.46 mg. per 100 gm. in mammary blood.	

The jugular blood contained 32.5 volumes per cent of corpuscles; the mammary, 30.9 volumes per cent.

shown when a trochar and cannula were inserted in her milk vein at 8.39 a.m. The cannula was left stoppered in the vein until 8.42 a.m., when about 575 cc. of blood were collected. The cow showed no sign of disturbance throughout this whole procedure. After the mammary blood had been collected, the cow's nose was secured and about 525 cc. of blood were collected from her jugular vein between 8.46 and 8.52 a.m. She was somewhat disturbed by this part of the procedure.

Both samples of blood were oxalated, and the greater part of each was centrifuged. The two samples of plasma were analyzed for total, lipoid, and inorganic phosphorus; the two portions of whole blood, which were not centrifuged, were analyzed for total and lipoid phosphorus.

Experiment 3. Comparison of Jugular and Mammary Blood and Plasma Obtained from Cow 17.—The animal calved normally on October 11, 1917, and, at the time of the experiment (November 1), was giving about 20 pounds of milk daily, with a fat content of 5.4 per cent. The calf had been kept with her dam and allowed to take milk from her at will from the time of her birth until the day of the experiment.

About 4.00 a.m., November 1, 1917, the calf was separated from her dam and kept without food. The cow, in the meantime, was allowed to stand in her stall, and disturbed as little as possible. At 10.50 a.m., a trochar and cannula were inserted in her milk vein as in the case of No. 211, Experiment 2. She moved a little when this was done, showing slightly more sign of pain than did Cow 211, but did not struggle or attempt to kick. The cannula was left stoppered in the vein, and the calf was brought in; the cow showed evident interest in the presence of her calf and stood quietly during the subsequent procedure. The calf was allowed to suck first for $1\frac{1}{2}$ minutes on the side opposite to where the cannula had been inserted in the vein, and then for $1\frac{1}{2}$ minutes, on the same side. 550 cc. of blood were then drawn while the calf was still sucking at intervals. The calf was somewhat disturbed by the presence of the experimenters and did not feed continuously but was actually sucking for about a quarter of the time that the mammary sample was being collected.

After the collection of the mammary sample, the cow's nose was secured, and 550 cc. of jugular blood were collected. The cow

was somewhat disturbed by this procedure, but much less than was No. 211 in Experiment 2. She stood quietly during the collection of the blood and chewed her cud a part of the time. During this part of the procedure, the calf sucked eagerly. Less than 5 minutes elapsed between the end of the collection of the mammary blood and the beginning of that of the jugular. The blood was treated and analyzed as in the preceding experiment.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.			
Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 10.43	10.55	6.01	5.27
II 10.17	10.77	Lipoid phosphorus decreased 0.74 mg. per 100 gm. in mammary plasma.	
III 9.98	10.87		
Average. 10.19	10.73		
Total phosphorus increased 0.54 mg. per 100 gm. in mammary plasma.			
Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations as compared with total.	
Jugular.	Mammary.	Jugular.	Mammary.
I 4.08	5.13	Lipoid... 6.01	5.27
II 4.12	5.25	Inorganic. 4.10	5.19
Average. 4.10	5.19	Sum..... 10.11	10.46
Inorganic phosphorus increased 1.09 mg. per 100 gm. in mammary plasma.		Total..... 10.19	10.73
Blood.			
Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 16.96	16.76	8.61	8.02
II 16.93	16.32	Lipoid phosphorus decreased 0.59 mg. per 100 gm. in mammary blood.	
Average. 16.94	16.54		
Total phosphorus decreased 0.40 mg. per 100 gm. in mammary blood.			

The jugular blood contained 31.4 volumes per cent of corpuscles; the mammary, 28.2 volumes per cent.

Experiment 4. Comparison of Jugular and Mammary Plasma and Blood Obtained from Cow 106.—The animal had calved on January 23, 1917, and had gone dry on December 30, 1917. She was due to calve again on February 17, 1918, but aborted on

Mg. per 100 Gm. of Plasma or Blood.

Plasma.					
Total phosphorus.			Lipoid phosphorus.		
Jugular.		Mammary.	Jugular.		Mammary.
I	9.41	10.27	4.91		4.89
II	9.69	10.31	Lipoid phosphorus decreased 0.02 mg. per 100 gm. in mammary plasma.		
III		10.37			
Average.	9.55	10.32			
Total phosphorus increased 0.77 mg. per 100 gm. in mammary plasma.			Sum of lipid and inorganic phosphorus concentrations as compared with total.		
Inorganic phosphorus.					
Jugular.		Mammary.	Jugular.		Mammary.
I	4.57	5.20	Lipoid....	4.91	4.89
II	4.53	5.07	Inorganic.	4.55	5.13
Average.	4.55	5.13	Sum.....	9.46	10.02
Inorganic phosphorus increased 0.58 mg. per 100 gm. in mammary plasma.			Total.....	9.55	10.32
Blood.					
Total phosphorus.			Lipoid phosphorus.		
Jugular.		Mammary.	Jugular.		Mammary.
I	14.94	15.16	7.92		7.77
II	14.57	14.86	Lipoid phosphorus decreased 0.15 mg. per 100 gm. in mammary blood.		
Average.	14.76	15.01			
Total phosphorus increased 0.25 mg. per 100 gm. in mammary blood.					

The jugular blood contained 31.4 volumes per cent of corpuscles; the mammary, 28.8 volumes per cent.

January 13, 1918, 9 days after the experiment, which was carried out January 4. Blood was obtained from her mammary and jugular veins essentially as in the case of No. 211, Experiment 2, the effort being made again to disturb her as little as possible. It was not so easy, however, to insert the trochar and cannula in her milk vein as it had been in the case of the milking cows, and she made two or three attempts to kick while this was being done. With this exception, the process of collecting blood ran about the same as in the case of Cow 211. It should be mentioned, however, that the blood ran decidedly less freely from the mammary cannula than in the case of the milking cows. 4 days after the experiment, it was found that milk could still easily be obtained from her udder. The blood was treated and analyzed as in the two preceding experiments.

Experiment 5. Comparison of Jugular and Mammary Plasma and Blood Obtained from Cow 115.—This animal was born November 7, 1914, and was, therefore, somewhat more than 3 years old on January 29, 1918, when the experiment was carried out. She had been bred at frequent intervals since August, 1916, but had never had a calf, and continued to come in heat frequently up to shortly before the time of the experiment. She was, therefore, probably not pregnant when the experiment was carried out. It was found possible, however, to get a few drops of milk from her udder on the day of the experiment. Her milk vein was much smaller than those of the milking cows or than those of the cows which were dry after a preceding period of lactation. Blood was obtained from her mammary and jugular veins essentially as in the case of Cows 211 and 106, Experiments 2 and 4, every effort being made to disturb her as little as possible by the experimental procedure. It was decidedly more difficult, however, to insert the mammary trochar and cannula than in any of the preceding experiments, and the cow made a number of vigorous attempts to kick while this was being done. The blood flowed less freely from the mammary cannula than in any of the preceding experiments. The jugular blood, however, was collected without any more difficulty than in the other experiments. The blood was treated and analyzed as in the two preceding experiments, except that the lipoid phosphorus was not determined in either the plasma or blood.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.			
Total phosphorus.		Inorganic phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 10.74	11.61	5.47	6.18
II 11.07	11.37	5.39	6.07
Average. 10.90	11.49	5.43	6.12
Total phosphorus increased 0.59 mg. per 100 gm. in mammary plasma.		Inorganic phosphorus increased 0.69 mg. per 100 gm. in mammary plasma.	

Blood.	
Total phosphorus.	
Jugular.	Mammary.
I 16.94	17.25
II 17.65	17.18
Average.....17.29	17.21
Total phosphorus decreased 0.08 mg. per 100 gm. in mammary blood.	

The jugular blood contained 37.8 volumes per cent of corpuscles; the mammary, 33.5 volumes per cent.

Experiment 6. Comparison of Plasma and Blood Obtained from Cow 9 before and after Disturbance.—The animal had her last calf on March 12, 1916, had gone dry September 10, 1917, and was probably not pregnant at time of the experiment, February 14, 1918. A trochar and cannula were inserted in her left jugular vein in the usual manner, and the cannula was left stoppered in the vein for half an hour. She was allowed to stand in her stall during this period, secured only by a halter, and was kept under observation. It was noticed that she stood quietly and gave no sign that she noticed the cannula in her neck. At the end of the half hour the cannula was unstoppered and 550 cc. of blood were drawn from it; this procedure caused less disturbance than did the insertion of the mammary trochar and cannula in the cases of Cows 211 and 17, Experiments 2 and 3. The cow's nose was then secured, another trochar and cannula were inserted in the right jugular vein, and 550 cc. of blood were obtained from it.

The disturbance caused by this part of the procedure was about the same as that caused by the corresponding procedure in Experiment 2 and rather more than in the case of Experiment 3. About 7 minutes elapsed between the collections of the two samples of blood. The blood was treated and analyzed as in Experiments 2, 3, and 4.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.					
Total phosphorus.			Lipoid phosphorus.		
First jugular.		Second jugular.	First jugular.		Second jugular.
I	9.92	9.90	4.71		4.66
II	10.29	10.18	4.84		4.84
Average.	10.10	10.04	4.77		4.75
0.06 mg. per 100 gm. difference.			0.02 mg. per 100 gm. difference.		
Inorganic phosphorus.			Sum of lipoid and inorganic phosphorus concentrations as compared with total.		
First jugular.		Second jugular.	First jugular.		Second jugular.
I	5.62	5.35	Lipoid....	4.77	4.75
II	5.55	5.39	Inorganic.	5.43	5.31
III	5.11	5.20			
Average.	5.43	5.31	Sum.....	10.20	10.06
0.12 mg. per 100 gm. difference.			Total.....	10.10	10.04
Blood.					
Total phosphorus.			Lipoid phosphorus.		
First jugular.		Second jugular.	First jugular.		Second jugular.
I	15.67	15.79			9.13
II	16.16	16.22	8.78*		8.92
Average.	15.91	16.00			9.02
0.09 mg. per 100 gm. difference.			0.24 mg. per 100 gm. difference.		

* Only one determination made.

The first jugular blood contained 29.3 volumes per cent of corpuscles; the second jugular, 29.8 volumes per cent.

Experiment 7. Effects of Feeding Calcium Chloride on the Calcium and Phosphorus Content of the Blood Plasma.—On January 9, 1917, a blood sample was obtained from Animal 17, a grade steer, about 5 months old and weighing about 250 pounds. Its daily ration for some time previously had been 14 pounds of skimmed milk, 3 pounds of grain, consisting of 375 parts cornmeal, 200 parts wheat bran, and 100 parts linseed oil meal, 1 pound of alfalfa hay, and 8 pounds of corn silage. It was kept on this ration throughout the experiment, except for the addition of calcium chloride. From January 9 to 11, 1.8 gm. of calcium, as calcium chloride, were fed daily with the milk; and from January 11 to 15, 4.7 gm. The feeding of the calcium chloride produced no perceptible bad effects. On January 15, a second blood sample was obtained.

The two samples of blood were citrated and centrifuged. The whole blood was analyzed for total phosphorus; the plasma, for total and inorganic phosphorus, and for calcium.

Mg. per 100 Gm. of Blood or Plasma.

Total phosphorus in whole blood.		Total phosphorus in plasma.	
Before calcium feeding.	After calcium feeding.	Before calcium feeding.	After calcium feeding.
I 19.65	18.38	10.80	9.56
II 19.21	18.66	10.64	10.42
Average. 19.43	18.52	10.72	9.99
Total phosphorus decreased 0.91 mg. per 100 gm. after calcium feeding.		Total phosphorus decreased 0.73 mg. per 100 gm. after calcium feeding.	
Inorganic phosphorus in plasma.		Calcium in plasma.	
Before calcium feeding.	After calcium feeding.	Before calcium feeding.	After calcium feeding.
I 7.44	7.14	11.24	11.48
II 7.34	6.82	10.77	11.19
Average. 7.39	6.98	11.00	11.33
Inorganic phosphorus decreased 0.41 mg. per 100 gm. after calcium feeding.		Calcium increased 0.33 mg. per 100 gm. after calcium feeding.	

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Experiment 8. Cow 19.—This experiment shows the changes which occur in the phosphorus content of the blood and plasma as the accompaniment of pregnancy and lactation. The subject was a grade Jersey about 8 years old, and was kept on a nearly

Experiment 8. Cow 19.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles, vol. per cent	Corpuscles, total P (mg. per 100 gm.).	Plasma.				Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1916								
Nov. 7	22.2*	39.3	33.8	13.9	8.5	5.9	1.35	Milking 229 days; 5 lbs. milk daily; pregnant, 154 days.
Dec. 22	18.8	40.8	28.8	11.1	5.7†	5.0	1.32	Milking 274 days; 2 lbs. milk daily; pregnant, 199 days.
1917								
Jan. 30	19.3‡	44.2	27.0	12.4	6.4†	6.0	1.32	Dry since Dec. 26, 1916; pregnant 238 days.
Mar. 9	19.5	42.1	29.9	11.0	5.0†	5.6	1.15	Pregnant 276 days.
Apr. 9	18.8	38.9	28.5	12.0	7.3†	4.9	1.25	Milking 28 days; 34.1 lbs. milk daily.
May 9	Not deter- mined.	32.5	—	15.7	8.7	6.8	1.27	Milking 58 days; 29 lbs. milk daily.
" 22	" "	32.5	—	13.3	8.1	4.9§	1.24	Milking 71 days; 28 lbs. milk daily.
June 22	20.7	37.8	30.7	14.0	8.7	4.6	1.32	Milking 102 days; 25 lbs. milk daily.

* Check 1, 21.4; check 2, 23.0; check 3, 22.3; all three used in calculating average.

† One determination only.

‡ Check 1, 18.8; check 2, 19.8.

§ Check 1, 4.4; check 2, 4.8; check 3, 5.4; all three used in calculating average.

|| Check 1, 4.3; check 2, 4.9; check 3, 4.5; all three used in calculating average.

constant ration from September 26, 1916, until July 9, 1917. From the former date until she calved on March 12, 1917, her daily ration was 10.8 pounds of grain (2 parts corn-meal, 1 part wheat bran, 1 part cottonseed meal), 7 pounds of alfalfa hay, 22 pounds of corn silage. For a few days immediately after she calved her ration was cut down, as is customary on most dairy farms; it was then resumed as above and continued until April 21, when her hay was reduced to 4 pounds daily, as she would not eat the 7 pounds which had previously been supplied. The hay was raised to 6 pounds on May 7, and this ration (10.8 pounds of grain as above, 6 pounds of alfalfa hay, 22 pounds of corn silage) was continued until July 9, 1917.

Experiment 9. Cow 33.—The latter part of this experiment is in general a repetition of Experiment 8, but with a lower ration. From April until November, 1917, the subject was kept on a nearly constant ration. She was fed daily 7 pounds of grain, consisting of 2 parts corn-meal, 2 parts wheat bran, and 1 part cottonseed meal, 6 pounds of alfalfa hay, and 25 pounds of corn silage. The small temporary changes which were made in this ration once or twice, on account of temporary loss of appetite or for other reasons, occurred at such times that they could have had no influence on the changes in the phosphorus content of the blood plasma which took place in connection with lactation and calving.

The first part of the experiment shows the changes in the phosphorus content of the blood and plasma which occurred as the accompaniment of a reduction in the rations. For 16 days before the first blood sample was obtained on September 27, 1916, her daily ration had been 7 pounds of grain (as above), 6 pounds of alfalfa hay, and 20 pounds of corn silage. From September 28 until November 2, inclusive, it was changed to 4 pounds of grain (as above), 4 pounds of alfalfa hay, and 25 pounds of corn silage. After November 2, it was restored to that fed before September 28.

Experiment 9. Cow 33.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.		Plasma.				Nitrogen (gm. per 100 cc.).	Remarks.
		vol. per cent	Corpuscles, total P (mg. per 100 gm.).	Phosphorus.					
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1916									
Sept. 27	20.3	34.6	32.5	13.3	8.6*	4.7†	1.21	Milking 42 days; 17.6 lbs. milk daily; high rations.	
Oct. 9	19.7	33.9	31.6	13.1	7.5‡	5.6	1.30	Milking 54 days; 15.4 lbs. milk daily; low rations since Sept. 28.	
" 19	20.4	33.5	34.6	12.7	7.1§	5.4	1.32	Milking 64 days; 15.7 lbs. milk daily; still on low rations.	
Nov. 2	19.3	34.6	30.0	13.2	7.7	4.6	1.29	Milking 78 days; 15 lbs. milk daily; still on low rations.	
Dec. 4	21.3	40.8	30.5	14.2¶	8.3§	5.4**	1.29	Milking 100 days; 16 lbs. milk daily; high rations since Nov. 3.	
1917									
Feb. 5	20.3	41.5	29.0	13.4	8.9§	4.3	1.33	Milking 163 days; 14.5 lbs. milk daily.	
Apr. 5	20.2	34.6	32.3	13.3	8.2‡‡	5.1	1.25	Milking 222 days; 12.2 lbs. milk daily.	
June 8	19.5	33.6	32.3	12.5	8.6‡‡	3.9	1.24	Milking 286 days; 12.9 lbs. milk daily.	
Aug. 3	19.1††	32.6	34.9	10.9	6.3‡‡	4.6	Not determined.	Dry since July 20; pregnant 284 days.	

Experiment 9. Cow 33—Concluded.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.			Nitrogen (gm. per 100 cc.).	Remarks.
				Phosphorus.				
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1917		vol. per cent						
Aug. 14	18.4	33.9	30.5	10.5§§	6.2††	4.3	Not determined.	Milking 8 days; 14 lbs. milk daily.
Oct. 6	19.6	33.5	32.7	12.5***	8.0§	4.2	" "	Milking 61 days; 15 lbs. milk daily.

* Check 1, 9.6; check 2, 8.6; check 3, 8.6; check 4, 8.6; checks 2, 3, and 4 used in calculating average.

† Calculated by subtracting lipid phosphorus from total.

‡ Figure based on one determination only; results of two other determinations were discarded, as they were obviously incorrect.

§ One determination only.

|| Check 1, 7.5; check 2, 8.0.

¶ Check 1, 14.4; check 2, 13.2; check 3, 14.0; checks 1 and 3 used in calculating average.

** Check 1, 5.6; check 2, 5.2; check 3, 5.4; all three used in calculating average.

†† Check 1, 19.6; check 2, 18.5.

‡‡ Calculated by subtracting inorganic phosphorus from total.

§§ Check 1, 11.2; check 2, 10.4; check 3, 10.8; check 4, 10.5; checks 2, 3, and 4 used in calculating average.

*** Check 1, 13.3; check 2, 12.2; check 3, 12.6; check 4, 12.4; checks 3 and 4 used in calculating average.

Experiment 10. Cow 39.—This experiment is, in general, a repetition of Experiment 9. Here, as in Experiment 9, the first part shows the effect of reducing the rations on the phosphorus content of the blood and plasma and on the milk yield, while the second part shows the changes in the phosphorus content of the blood and plasma which occur as the accompaniment of pregnancy and lactation on a nearly constant ration.

From September 1 to 25, 1916, this cow was fed daily 6 pounds of grain (same as that fed to No. 33), 6 pounds of alfalfa hay, 15 pounds of corn silage. From September 26 until November 2, she was fed 3 pounds of grain (as above), 3 pounds of alfalfa hay,

Experiment 10. Cow 39.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.			Nitrogen (gm. per 100 cc.).	Remarks.
				Phosphorus.				
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1916		vol. per cent						
Sept. 23	19.8	30.4	30.7	14.8	7.1*	7.7†	1.13	Milking 117 days; 14.2 lbs. milk daily; high rations.
Oct. 5	18.2	29.1	29.8	13.2	7.0*	6.2†	1.15	Milking 129 days; 10.6 lbs. milk daily; low rations since Sept. 25.
" 16	17.1*	29.3	28.6	12.1	6.4*	5.1	1.13	Milking 140 days; 11.2 lbs. milk daily; still on low rations.
" 30	15.8	29.2	26.4	11.2	6.5	4.6‡	1.14	Milking 154 days; 9.5 lbs. milk daily; still on low rations.
Nov. 27	19.0	34.6	28.8	13.4	7.7	5.7	1.16	Milking 182 days; 12.7 lbs. milk daily; high rations since Nov. 3.
1917								
Jan. 23	17.1	33.9	26.2	12.1	6.8*	4.6§	1.16	Milking 239 days; 10.3 lbs. milk daily.
Mar. 26	16.9	38.9	24.6	11.5	6.4	5.3*	1.15	Milking 301 days; 5.8 lbs. milk daily.
May 28	17.3	38.9	25.4	11.6	5.6	6.0	1.17	Dry since May 18; pregnant 230 days.
July 24	16.3	32.5	27.1	10.7	6.5	4.2	1.13	Pregnant 287 days.
Aug. 9	16.0	30.4	27.4	10.7	6.5	4.2	Not determined.	Milking 12 days; 20.2 lbs. milk daily.
Oct. 2	19.1	31.4	29.3	14.2	8.9	5.3	" "	Milking 66 days; 17.0 lbs. milk daily

* One determination only.

† Calculated by subtracting lipoid phosphorus from total.

‡ Check 1, 4.6; check 2, 3.9; check 3, 4.5; checks 1 and 3 used in calculating average.

§ Check 1, 4.8; check 2, 4.5.

|| Calculated by subtracting inorganic phosphorus from total.

and 25 pounds of corn silage. After November 2 the ration was restored to that fed before September 25, and this ration was kept up (except for a reduction in the hay between April 21 and 30, 1917, on account of temporary loss of appetite) until she calved on July 28, 1917. It was cut down as usual for a few days after calving, then made 6 pounds of grain (as above), 6 pounds of alfalfa hay, and 30 pounds of corn silage. On September 10 the grain was increased to 8 pounds, and the silage was reduced to 25; this ration was continued until after October 2, 1917.

Experiments 11, 12, 13, and 14. Heifers 79, 81, 119, and 120.—These experiments show, in the first place, the changes which occur in the concentrations of various constituents of the blood and plasma as the result of advancing age. In the second place, they throw some light on the changes which can be produced in the concentrations of calcium and inorganic phosphate in the plasma by feeding either calcium chloride or sodium phosphate.

The four heifers, which served as subjects, were fed in general, as nearly as possible alike. They received rations like those recommended in the standard books on the subject, the object being to keep them growing and in good condition. This object was attained in all four cases.

The following is a rough description of the manner of feeding. 10 pounds of whole milk were fed daily up to the 10th day. Skimmed milk was then gradually substituted, and the calves got 12 pounds of this daily up to the 30th day. From the 30th to the 180th day the daily milk ration was 14 pounds; it was then rapidly reduced, and no more milk was fed after the 200th day. Small amounts of alfalfa hay were offered (and usually not eaten at first) from the 7th day on. From the end of the 3rd week small amounts of grain (375 parts corn and cob meal, 200 parts wheat bran, 100 parts linseed oil meal) and corn silage were also offered. When 60 days old the heifers were usually eating about a pound of grain, half a pound of hay, and 3 pounds of silage daily besides the milk. The grain, hay, and silage were gradually increased until at 300 days the calves were eating 3 pounds of grain, 3 pounds of alfalfa hay, and about 25 pounds of corn silage daily. The grain and hay were not increased beyond this point, but the silage was increased in some cases to as much as 32 pounds. Records were kept of the amounts of the various feeds eaten in each case. All the calves took practically all the milk and grain that was offered to them, so that the rations were nearly

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similar in all four cases as far as these constituents were concerned. They also took all the hay after the first 4 or 5 weeks. There was some variation in the amount of silage eaten, but this was so small that the experiments may be regarded as comparable as far as the basic rations were concerned.

Heifers 79 and 120 were fed on the rations which have just been described, without additions. Heifer 81 received daily doses of calcium chloride in addition; and Heifer 119, daily doses of disodium phosphate. From February 20 to July 25, 1917, Heifer 81 received 2.6 gm. of Ca daily as calcium chloride; and from July 25 on, 5.7 gm. of Ca. From January 11 to March 19, 1917, Heifer 119 received 4.5 gm. of P daily as Na_2HPO_4 , and from March 19 on, 6.9 gm. daily.

Heifer 119 showed no bad effects whatever as the result of the phosphate feeding, and Heifer 81 also showed no bad effects from the calcium feeding.

Experiment 11. Heifer 79, Fed Basic Ration without Addition.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1916		vol. per cent							
Dec. 12	31.5	49.5	52.0	8.5	2.0*	6.3	0.959	11.2*	1 day old; born Dec. 11.
1917									
Feb. 17	20.0†	43.1	32.3	9.5	3.1*	6.4*	1.164	10.1	68 days old.
Apr. 16	22.0	42.0	32.8	13.2‡	4.2§	9.0	1.070	10.0	126 " "
July 19	20.9	34.6	33.6	13.6	6.9§	6.7	1.091	9.4	220 " "
Nov. 26	22.0	39.9	32.2	14.5	6.2§	8.3	Not determined.	10.1	350 " "
1918									
Apr. 29	17.9	37.8	27.6	11.4	5.8§	5.6	" "	9.4	1 yr., 139 days old.

* One determination only.

† Check 1, 20.8; check 2, 19.2.

‡ Check 1, 12.1; check 2, 13.2; check 3, 13.3; checks 2 and 3 used in calculating average.

§ Calculated by subtracting inorganic from total.

|| Check 1, 10.5; check 2, 10.1; check 3, 10.0; check 4, 9.8; all four used in calculating average.

Experiment 12. Heifer 81, Fed Basic Ration plus Calcium Chloride.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles. vol. per cent	Corpuscles, total P (mg. per 100 gm.).	Plasma.						Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).		
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).				
1917										
Feb. 20	29.5*	34.6	63.8	9.6†	2.6	6.0‡	1.148§	13.4	1 day old; born Feb. 19.	
Apr. 20	17.5	31.4	39.5	9.4	2.2	7.2	1.038	10.4	60 days old.	
June 19	18.3	34.1	29.7	11.9	4.9	7.0¶	0.918	10.6	120 " "	
Sept. 22	19.0	38.9	28.9	12.0	4.1	7.9	Not determined.	10.5	215 " "	
1918										
Jan. 24	17.9	37.8	28.1	11.1	4.4	6.7	" "	9.7	339 " "	

* Figure based on one determination only; the result of a second determination was discarded as it was obviously incorrect.

† First two determinations discarded as they did not agree with one another; the figure given is based on another pair which was 9.74 and 9.46 respectively.

‡ Check 1, 6.3; check 2, 5.8.

§ Check 1, 1.112; check 2, 1.184.

|| Calculated by subtracting inorganic from total.

¶ Check 1, 7.4; check 2, 6.7

Experiment 13. Heifer 119, Fed Basic Ration plus Disodium Phosphate.

Date.	Blood, total P (mg. per. 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.):	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1918		vol. per cent							
Jan. 11	28.1	36.7	56.4	10.0*	3.5†	6.3‡	1.146§	13.7	2 days old, born Jan. 9.
Mar. 12	20.8	36.7	34.6	12.0¶	3.3†	8.8‡	0.975	10.8	62 days old.
May 14	21.2	41.5	33.2	11.7	3.2**	8.5	1.018	10.5	125 " "
Aug. 23	20.0	33.0	33.2	13.0	5.0**	8.0	Not determined.	10.1	226 " "
Dec. 3	21.6	41.5	31.3	13.9	6.5**	7.4	" "	10.8	328 " "

* Check 1, 11.1; check 2, 10.1; check 3, 9.9; checks 2 and 3 used in calculating average.

† One determination only.

‡ Check 1, 6.9; check 2, 5.9; check 3, 6.5; check 4, 6.2; checks 3 and 4 used in calculating average.

§ Check 1, 1.190; check 2, 1.102.

|| Check 1, 21.6; check 2, 20.0.

¶ Check 1, 12.4; check 2, 12.1; check 3, 11.5; all three used in calculating average.

** Calculated by subtracting inorganic from total.

Experiment 14. Heifer 120, Fed Basic Ration without Addition.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1917		vol. per cent							
Apr. 24	17.7	33.0	35.1	8.4*	2.2†	6.2	1.052	9.7	62 days old, born Feb. 21.
June 29	19.7	38.9	30.6	12.0	5.4†	6.6	1.059	10.1	128 days old.
Sept. 27	22.0†	34.6	33.4	15.5	6.3†	9.2	Not determined.	9.1	218 " "
1918									
Mar. 4	18.4	35.1	29.1	12.1	4.9†	7.2	" "	9.8	1 yr., 11 days old.

* Check 1, 8.6; check 2, 7.6; check 3, 8.2; checks 1 and 3 used in calculating average.

† Calculated by subtracting inorganic from total.

‡ Check 1, 22.8; check 2, 21.1.

Experiment 15. Heifer 214.—This experiment throws some further light on the changes in the calcium and phosphorus content of the blood and plasma which occur as the accompaniment of increasing age, of pregnancy, and of changes in ration.

The subject was a thoroughbred Holstein, born March 18, 1916. Up to August 4, 1917, no particular effort was made to control her rations, and she was fed about as the calves are usually fed at Beltsville, but with considerably more skimmed milk than was given to Heifers 79, 81, 119, and 120. From August 4 to September 10, 1917, she was fed daily 3 pounds of grain, consisting of 2½ parts corn and cob meal, 2 parts wheat bran, 1 part cottonseed meal; 4 pounds of alfalfa hay; 15 pounds of corn silage. She became pregnant on August 23. On September 10, her silage was raised to 25 pounds.

On December 18, an experiment was started, one of the objects of which was to gain further light on the possibility of raising the concentration of inorganic phosphate in the blood plasma by feeding sodium phosphate. From this date until January 18, 1918, the heifer was fed 3 pounds of grain (as above), 4 pounds

Experiment 15. Heifer 214.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.		Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
					Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
					Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
<i>1916</i>		<i>vol. per cent</i>								
May 11	18.0	28.2	38.9	9.2		Not determined.	Not determined.	1.02	11.4	54 days old; born Mar. 18.
July 12	21.7	43.7	32.1	12.6		" "	" "	1.00	10.9	116 days old.
Aug. 21	21.4	44.9	30.2	13.2		" "	" "	0.98	10.6	156 " "
Nov. 20	19.6	30.5	34.6	12.7		4.7*	7.8	0.97	10.3	247 " "
<i>1917</i>										
Jan. 18	20.5	37.3	31.8	13.2		5.4†	6.1	1.03	10.7	306 " "
Mar. 16	18.7	42.8	27.5	11.4		4.9*	6.4	1.09	10.2	363 " "
May 17	18.7	35.1	30.1	12.0		5.8†	6.2	1.12	10.8	1 yr., 60 days old.
Aug. 17	16.8	31.1	31.0	10.0		4.2†	5.8	Not determined.	9.7	1 yr., 152 days old.
Sept. 10	17.1§	35.7	30.4	9.0		4.1†	4.9	" "	10.3	Pregnant 18 days; 1 yr., 176 days old.
Oct. 11	17.5	34.1	29.5	10.8		5.4*	5.3	" "	10.4	Pregnant 49 days; 1 yr., 207 days old.
Nov. 16	17.6	38.8	28.6	9.9*		5.1*	5.1	" "	10.5*	Pregnant 85 days; 1 yr., 243 days old.
Dec. 10	18.2	41.0	28.8	10.0		4.5†	5.5*	" "	11.1	Pregnant 109 days; 1 yr., 267 days old.
<i>1918</i>										
Jan. 11	16.4	35.1	28.3	9.4		4.4†	5.0	" "	10.3	Pregnant 141 days; 1 yr., 299 days old.

Experiment 15. Heifer 214—Concluded.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1918		vol. per cent							
Feb. 8	18.8	37.8	28.7	12.2	3.9†	8.3	Not determined.	10.0	Pregnant 169 days; 1 yr., 327 days old; phosphate since Jan. 19.
Mar. 20	17.1	36.7	28.4	9.9	4.4‡	5.5	" "	9.6	Pregnant 209 days; 2 yrs., 2 days old; no phosphate since Feb. 27.
May 2	15.6	37.8	24.1	9.9	5.9‡	4.0	" "	Not determined.	Pregnant 252 days; 2 yrs., 45 days old; phosphate since Mar. 29.

* One determination only.

† Check 1, 5.5; check 2, 5.5; check 3, 4.8; check 4, 5.3; checks 1, 2, and 4 used in calculating average.

‡ Calculated by subtracting inorganic from total.

§ Check 1, 14.8; check 2, 17.3; check 3, 16.9; check 4, 16.9; checks 2, 3, and 4 used in calculating average.

of alfalfa hay, 25 pounds of corn silage. January 19 to February 26, 1918, she was fed hay and grain on alternate days, and disodium phosphate was added to the grain, the manner of feeding being as follows. 1 day, 6 pounds grain (as above), but with 22 gm. of phosphorus as Na_2HPO_4 added to it; 25 pounds of corn silage; the next day, 8 pounds of alfalfa hay, 25 pounds of corn silage. From February 27 to March 27, this manner of feeding was continued, but the grain was fed without the phosphate and the silage was raised to 30 pounds.



THE PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF HUMAN AMNIOTIC FLUID.

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An inquiry into the biological meaning of the human amniotic fluid demands, first of all, a sufficient knowledge of its physical properties and chemical composition, information which many investigators have attempted to gain. Prochownick (1) studied the sodium chloride, solid substances, and urea in the amniotic fluid; Schroeder (2) determined the quantities of solid substances, ash, and albumins; Farkas and Scipiades (3) inquired into its physical properties and into the quantity of sodium chloride contained in it; Jacqué (4) and Grünbaum (5) measured its freezing point. These reports, however, are defective either in the methods used, or in the statement of their examinations; accordingly, in many cases, the results are highly problematical. Döderlein (6) and Jacqué (4) performed some fairly exact analyses of amniotic fluid of mammalia, but their results cannot be applied directly to human amniotic fluid.

Physical Properties of Human Amniotic Fluid.

A study of the literature of the amniotic fluid may be said to establish the following facts: the fluid has a specific gravity varying from 1.002 to 1.028; a freezing point which varies from $\Delta = -0.45$ to -0.5° ; it is always hypotonic against the blood of the maternal body and the fetus. On the basis of these facts, most investigators have concluded that the amniotic fluid is not a simple transudate, but a mixture of fetal urine with maternal transudate.

Experimental Material.

The urine of women in labor was removed through a Nelaton's catheter after disinfection of the vulva; a sterile dry cylindrical speculum was in-

roduced deep into the vagina, and the os uteri and its circumference were wiped with sterile gauze, thereby cleansing it entirely from blood, mucous particles, etc. The fetal membranes were pierced with a sharp instrument, and the amniotic fluid flowing out from the inside of the womb was collected in a clean glass flask, and preserved with a small quantity of toluene in an ice box. Thus obtained, the amniotic fluid is almost pure, since it has little chance to become impure, as it flows along the sides of the speculum, without touching the mucous membrane of the vagina or the skin of the vulva.

The amniotic fluid is slimy, yellowish white or pale yellow, and cloudy like soap water (rarely clear); it almost always contains much mucous flocks and has a peculiar odor. Because of its sliminess, the fluid is first filtered through fourfold gauze and later with suction. Thus prepared, the slightly cloudy liquor was used for the following experiments.

Specific Gravity.

With a 5 or 10 cc. pyknometer, the specific gravity of the amniotic fluid of women at the end of pregnancy was determined in twenty-three cases, and was found to average 1.0078. In six cases, the liquor was yellowish green, mixed with meconium, with a specific gravity varying from 1.0081 to 1.0136, averaging 1.0106; in the remaining seventeen cases, the liquor was yellowish white or yellow, and the specific gravity varied from 1.0046 to 1.0099, averaging 1.0069. Furthermore, in one case in the 5th month of pregnancy, in which artificial abortion was performed because of tuberculosis, the specific gravity of the fluid was 1.008, and in another case at the beginning of the 6th month, it was 1.0087. The admixture of meconium always causes an increase of the specific gravity. In one instance, in the first half of pregnancy, no evident difference was found.

The relation between the specific gravity and the freezing point is shown in Table I.

Freezing Point and Osmotic Pressure.

By use of Beckmann's cryoscopic apparatus, the freezing point of the amniotic fluid of twenty-two women at the end of pregnancy was determined. The value of Δ was found to vary from 0.46 to 0.565°, with an average of 0.504°. In sixteen cases, the amniotic fluid was yellowish or yellowish white, with an average freezing point of 0.504°; in the other six cases, the liquor was yellowish green, mixed with meconium, and had an average freez-

TABLE I.

No. of amniotic fluid.	Specific gravity.	Temperature of fluid.		Freezing point (Δ).	$C = \frac{\Delta}{1.88}$	Osmotic pressure in atmospheres (at 0°C).	New-born child.			Remarks.
		°C.	°C.				Sex.	Length.	Weight.	
								cm.	gm.	
1	1.0066	25	0.485	0.260	5.837	F.		49	2,500	Asphyxia of second degree.
2	1.0072	21	—	—	—	M.		47	2,460	
5	1.0065	19	0.498	0.267	5.993	F.		50	2,500	
7	1.0076	18	0.480	0.258	5.777	M.		46	2,150	
8	1.0067	19	0.565	0.303	6.800	F.		50	3,000	
10	1.0083	19	0.563	0.302	6.776	M.		54	3,200	
12	1.0071	19	0.500	0.268	6.017	"		48	2,780	
13	1.0065	19	0.475	0.255	5.716	F.		49	3,000	
14	1.0066	19	0.500	0.268	6.017	"		47	3,300	
16	1.0073	19	0.490	0.263	5.897	M.		50	3,110	
18	1.0065	19	0.460	0.247	5.536	F.		49	2,600	
19	1.0082	17	0.520	0.279	6.258	M.		53	3,050	
20	1.0075	17	0.525	0.282	6.318	"		49	2,500	
22	1.0066	18	0.512	0.275	6.162	F.		41	2,070	
23	1.0064	17	0.505	0.271	6.077	M.		52	3,420	
24	1.0074	17	0.500	0.268	6.017	"		49	2,970	
27	1.0046	18	0.495	0.266	5.957	F.		47	3,050	
Average..	1.0069		0.504	0.271	6.072	—		—	—	
4	1.0136	21	0.565	0.303	6.800	M.		46	2,000	Albuminuria, beri-beri, still-born, mixed with meconium. With meconium. " " " " " "
6	1.0123	18	0.518	0.278	6.234	"		50	2,950	
9	1.0090	19	0.478	0.256	5.753	"		52	2,810	
11	1.0099	19	0.513	0.275	6.174	"		54	2,800	
17	1.0108	19	0.488	0.262	5.873	"		48	2,680	
21	1.0081	17	0.470	0.252	5.656	"		55	3,150	
Average..	1.0106		0.505	0.271	6.081	—		—	—	
191	1.0080	34	—	—	—					5th month.
227	1.0087	18	0.540	0.293	6.499					6th "

ing point of 0.505°. According to these results, it seems that the admixture of meconium has no marked influence upon the freezing point. The amniotic fluid of a pregnant woman at the beginning of the 6th month showed the freezing point Δ to be 0.54°.

From the formulas $P = 0.082 CT$ (in which P = osmotic pressure, C = osmotic concentration, and T = absolute temperature) and $C = \frac{\Delta}{E}$ (in which Δ = the lowering of freezing point, and E = a constant), the osmotic pressure of the liquor at 0° was calculated (Table I). The values for the sixteen cases of pure amniotic fluid varied from 5.536 to 6.80 atmospheres, with an average of 6.072, while those of the six cases in which the fluid was mixed with meconium varied from 5.656 to 6.80, with an average of 6.081 atmospheres.

Specific Electrical Conductivity.

The resistance of twenty-three samples of amniotic fluid was measured by the usual method, and the specific electrical conductivity calculated from the formula $U = \frac{C}{W}$ (W = resistance, C = resistance capacity). The resistance capacity was determined by using a 0.1 N KCl solution. In seventeen cases, the amniotic fluid was yellowish and the specific electrical conductivity varied from 119.06×10^{-4} to 134.00×10^{-4} , with an average of 127.15×10^{-4} . In six cases, the fluid was yellowish green, mixed with meconium, and the specific electrical conductivity varied from 120.62×10^{-4} to 132.84×10^{-4} , with an average of 126.90×10^{-4} . The admixture of meconium seems to have no marked influence on the electrical conductivity.

Hydrogen Ion Concentration.

According to Hasselbalch's (7) method, a gas electrode was made with amniotic fluid, and connected with a calomel electrode (0.3377 volt), an accumulator whose electromotive force was previously determined in comparison with a cadmium standard cell, a resistance bridge, and a capillary electrometer; the electromotive force of the fluid was determined, and from this the H ion concentration was calculated. The results with the H ion concentration are shown in Table II.

In sixteen cases, the amniotic fluid was pure, and the H ion concentration varied from 0.2266×10^{-8} to 0.2648×10^{-7} , with an average of 0.1282×10^{-7} . In five cases, the liquor was yel-

TABLE II.

No. of amniotic fluid.	Specific electrical conductivity at 25°.	Reaction (litmus).	H ion concentration.	Temperature. °C.	Remarks.
1	124.43×10^{-4}	Weakly alkaline.	0.4845×10^{-8}	25	
2	$128.90 \times$				
5	$128.90 \times$	Weakly alkaline.	$0.7189 \times$	19	
7	$127.84 \times$	" "	$0.9270 \times$	19	
8	$129.11 \times$	" "	$0.2266 \times$	18	
10	$122.11 \times$	Neutral.	$0.3207 \times$	19	
12	$130.55 \times$	"	$0.5251 \times$	19	
13	$124.64 \times$	"	0.2269×10^{-7}	19	
14	$125.34 \times$	Weakly alkaline.	$0.2536 \times$	19	
16	$128.89 \times$	Neutral.	$0.2062 \times$	19	
18	$119.09 \times$	Weakly alkaline.	0.9804×10^{-8}	17	
19	$134.00 \times$	" "	0.2583×10^{-7}	17	
20	$134.00 \times$	" "	0.5002×10^{-8}	17	
22	$133.22 \times$	" "	$0.8628 \times$	18	
23	$127.40 \times$	" "	0.2648×10^{-7}	18	
24	$122.54 \times$	Neutral.	$0.1449 \times$	18	
27	$120.69 \times$	"	$0.1432 \times$	18	
Average.	$127.15 \times$		$0.1282 \times$		
4	$132.84 \times$	Weakly alkaline.	0.8338×10^{-8}	21	Still-born, mixed with meconium.
6	$127.97 \times$	" "	$0.7189 \times$	19	Mixed with meconium.
9	$120.62 \times$	Neutral.	0.1453×10^{-7}	19	Mixed with meconium.
11	$130.14 \times$	"	0.6131×10^{-8}	19	Mixed with meconium.
17	$126.74 \times$	Weakly alkaline.	$0.8329 \times$	19	Mixed with meconium.
21	$123.14 \times$	Neutral.			Mixed with meconium.
Average.	$126.90 \times$		0.8903×10^{-8}		

lowish green, mixed with meconium, and the H ion concentration varied from 0.6131×10^{-8} to 0.1453×10^{-7} , with an average of 0.8903×10^{-8} . It is evident from these figures that the reaction of the amniotic fluid is slightly alkaline. In addition, the reaction was tested with litmus paper; in thirteen cases, the reaction was weakly alkaline, in nine cases it was neutral; in no case was the reaction acid.

Optical Activity.

The albumin was coagulated by adding a small quantity of sodium chloride, making the solution slightly acid with 1 per cent acetic acid, and heated on a water bath. After filtering, only one of the twenty-two samples examined showed any optical activity (-0.01°).

Summary.—(1) The specific gravity of pure amniotic fluid averages 1.0069 and never rises higher than 1.010. (2) The freezing point averages 0.504° ; the difference between the greatest and the smallest amounts to nearly 0.1° . The osmotic pressure averages 6.072 atmospheres. (3) The specific electrical conductivity averages 127.15×10^{-4} , and corresponds nearly to that of a 0.1 N solution of KCl. (4) The average H ion concentration is 0.1282×10^{-7} ; that is, slightly alkaline. (5) The reaction with litmus paper is either slightly alkaline or neutral. (6) It is nearly optically inactive.

Inorganic Components of Human Amniotic Fluid.

Farkas and Scipiades (3) found 0.444 to 0.58 per cent (average 0.507 per cent) sodium chloride in the amniotic fluid of five pregnant women. Jacqu  (4) analyzed the amniotic fluid of eighteen sheep fetuses with a length of 2 to 49 cm. and found: insoluble ash 0.017 per cent, soluble ash, 0.82 per cent, sodium chloride 0.64 per cent, and total ash 0.84 per cent. He also analyzed the fluid of two swine and found: insoluble ash 0.024 to 0.030 per cent, soluble ash 0.74 to 0.76 per cent, sodium chloride 0.53 to 0.55 per cent, and total ash 0.77 to 0.78 per cent. D derlein (6) performed exact analyses of the amniotic fluid of fifteen cows and found: Cl 0.358 per cent, NaCl 0.586 per cent, Na_2O 0.367 per cent, K_2O , 0.060 per cent, Ca 0.014 per cent, Mg 0.0038 per cent. Nauta (8) found 0.36 per cent chlorine, 1.44 per cent solid substances, and 0.59 per cent ash in the amniotic fluid of cows.

Quantitative Determination of Water, Solid Substances, Ash, and Organic Substances.

The filtered amniotic fluid was centrifuged for 30 minutes, freed from sediment, and filtered with suction. A known quantity of this filtrate, which in most cases was still a little cloudy, was evaporated on a water bath, and dried to constant weight

at 110°C., giving the solid substance. This was then ashed and separated into soluble and insoluble ash. The sum of the two is total ash; the organic substances and the water were obtained by difference. The results of the analyses are shown in Tables III to VI.

TABLE III.

Amniotic fluid.	Average.	High.	Low.
	Gm. per 100 cc. fluid.		
Water (15 cases).....	99.49	99.76	99.29
Solid substances (26 cases).....	1.24	1.53	1.02
Soluble ash (20 cases).....	0.77	0.85	0.72
Insoluble ash (20 cases).....	0.04	0.05	0.03
Total ash (20 cases).....	0.81	0.90	0.76
Organic substances (20 cases).....	0.45	0.72	0.26

TABLE IV.

No. of amniotic fluid.	Quantity of amniotic fluid.	Solid substances per 100 cc.	Water per 100 cc.	Remarks.
A.				
	cc.	gm.	gm.	
1	20	1.11	99.55	
5	20	1.11	99.54	
7	15	1.27	99.49	
12	20	1.20	99.51	
13	20	1.14	99.51	
14	15	1.26	99.40	
Average.		1.18	99.50	
B.*				
4	20	1.73	99.63	Mixed with meconium.
6	10	1.84	99.39	" " "
9	20	1.77	99.13	" " "
11	20	1.95	99.04	" " "
Average.		1.82	99.30	

* Excluded from the average.

TABLE V.

No. of amniotic fluid.	Quantity of amniotic fluid. cc.	Solid substances.	Soluble ash.	Insoluble ash.	Total ash.	Organic substances.	Water.	Remarks.	
		Gm. per 100 cc. fluid.							
16	25	1.19	0.75	0.04	0.79	0.40	99.54	Mixed with a little meconium.	
17	20	1.32	0.72	0.04	0.76	0.56	99.76		
18	20	1.02	0.72	0.04	0.76	0.27	99.63		
19	20	1.53	0.77	0.04	0.81	0.72	99.29		
20	20	1.25	0.78	0.04	0.82	0.43	99.50		
22	15	1.23	0.80	0.04	0.84	0.39	99.43		
23	25	1.15	0.74	0.04	0.78	0.36	99.49		
24	20	1.35	0.75	0.04	0.78	0.57	99.39		
27	20	1.13	0.72	0.04	0.76	0.37	99.33		
29	15	1.41	0.81	0.04	0.86	0.55			
Average.		1.26	0.76	0.04	0.80	0.46	99.48		

TABLE VI.

No. of amniotic fluid.	Quantity of amniotic fluid.	Solid substances.	Soluble ash.	Insoluble ash.	Total ash.	Organic substances.	Remarks.	
		Gm. per 100 cc. fluid.						
30	20	1.27	0.76	0.04	0.80	0.46	Mixed with a little meconium.	
35	20	1.24	0.79	0.03	0.82	0.42		
37	20	1.34	0.73	0.05	0.78	0.56		
39	20	1.16	0.78	0.05	0.83	0.33		
40	20	1.07	0.72	0.04	0.76	0.31		
41	20	1.42	0.84	0.04	0.88	0.54		
44	20	1.14	0.79	0.03	0.82	0.32		
47	20	1.50	0.76	0.04	0.79	0.70		
49	20	1.22	0.80	0.04	0.84	0.38		
50	20	1.26	0.85	0.04	0.90	0.36		
Average.		1.26	0.78	0.04	0.82	0.44		

Quantitative Determination of Inorganic Components.

The soluble ash was dissolved in water; chlorine, sulfuric acid, phosphoric acid, potassium, sodium, calcium, and magnesium were determined. The insoluble ash was dissolved in hydrochloric acid, then diluted with water, and used for the determinations of sulfuric acid, phosphoric acid, calcium, and magnesium.

Chlorine was precipitated and weighed as silver chloride. Sulfuric acid was precipitated as barium sulfate. Phosphoric acid was determined in the aqueous solution or hydrochloric acid solution by Neumann's (9) method.

Potassium and Sodium.—Phosphoric acid and sulfuric acid were removed as insoluble barium salts; ammonium carbonate solution and ammonia were added to the filtrate to precipitate the excess barium; the filtrate was evaporated after adding a drop of hydrochloric acid; the ammonium carbonate and ammonium chloride were removed by heating, and the residue was weighed as total alkali chlorides. The quantity of potassium in these alkali chlorides was determined as potassium platonic chloride, and the quantity of sodium determined by difference.

Calcium.—The aqueous or hydrochloric acid solution was treated with liquid ammonia till it reacted strongly alkaline; it was then made acid with acetic acid, and the precipitate of iron salts filtered. The filtrate was warmed on a water bath and, after addition of ammonium oxalate solution, was further warmed until the calcium oxalate was entirely precipitated. The precipitate of calcium oxalate was heated and weighed as calcium oxide.

Magnesium.—The filtrate from the calcium oxalate was concentrated, ammonia added till it reacted strongly alkaline, and sodium citrate and sodium phosphate were added. The precipitate of calcium magnesium phosphate was converted into magnesium pyrophosphate and weighed.

Iron was determined by Neumann's (10) method.

A preliminary qualitative analysis indicated the presence in the soluble ash of amniotic fluid of chlorine, sulfuric acid, phosphoric acid, Na, K, Ca, and Mg, and in the insoluble ash of carbonic acid, sulfuric acid, phosphoric acid, Ca, Mg, and Fe.

The 200 cc. of amniotic fluid examined in Table IV, and those in Tables V and VI were quantitatively analyzed, with results as shown in Table VII.

For a fourth sample, 10, 20, and 170 cc. of amniotic fluid were taken respectively from three women—200 cc. in all— and for a fifth sample, 60,

TABLE VII.

	Amniotic fluid (200 cc.) (Table V).				Amniotic fluid (200 cc.) (Table VI).			
	Soluble ash (1.563 gm.).	Insoluble ash (0.0622 gm.).	Total ash (1.5375 gm.).	Ash per 100 cc. fluid.	Soluble ash (1.561 gm.).	Insoluble ash (0.0607 gm.).	Total ash (1.6458 gm.).	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Cl.....	0.7313	0	0.7313	0.3656	0.7377	0	0.7377	0.3688
NaCl*.....			1.2050	0.6025			1.2154	0.6077
SO ₃	0.1056	Trace.	0.1056	0.0528	0.1490	Trace.	0.1490	0.0745
S.....	0.0422	"	0.0422	0.0211			0.0596	0.0298
P ₂ O ₅	0.0048	0.0082	0.0130	0.0065	0.0027	0.0094	0.0121	0.0060
P.....			0.0056	0.0028	0.0011	0.0041	0.0052	0.0026
Na ₂ O.....	0.7531	0	0.7531	0.3765	0.7661	0	0.7661	0.3830
Na.....	0.5587	0	0.5587	0.2793	0.5685	0	0.5685	0.2842
K ₂ O.....	0.0489	0	0.0489	0.0244	0.0464	0	0.0464	0.0232
K.....	0.0408	0	0.0408	0.0204	0.0385	0	0.0385	0.0192
CaO.....	0.0030	0.0314	0.0344	0.0172	0.0024	0.0302	0.0326	0.0163
Ca.....	0.0021	0.0224	0.0245	0.0122	0.0017	0.0215	0.0232	0.0116
MgO.....	Trace.	0.0008	0.0008	0.0004	0.0010	0.0002	0.0012	0.0006
Mg.....	"	0.0004	0.0004	0.0002	0.0006	0.0001	0.0007	0.0003

* The quantity of NaCl was calculated from the quantity of Cl.

40, and 100 cc. were taken. After complete evaporation and conversion into ash, quantitative analyses were performed, with results as in Table VIII.

Examining the above figures, we find that the only marked variation is in the amount of sulfuric acid.

To find the relations of the albumins in amniotic fluid to sulfuric acid and other ash, 400 cc. of the fluid obtained from fourteen cases were divided into two equal parts. The one part (A) was dried, ashed, dissolved in water, and diluted up to 200 cc., of which 20 cc. were used for the quantitative determination of sulfuric acid, 50 cc. for phosphoric acid, and 130 cc. for Ca and Mg. The other part (B) was diluted with nine volumes of water, and the albumins were entirely precipitated with common salt and colloidal ferric hydroxide according to Rona and Michaelis (11) and Oppler and Rona. The precipitate was mixed with 500 cc. of water and filtered; this treatment was repeated two more

TABLE VIII.

	I. Amniotic fluid (200 cc.).				II. Amniotic fluid (200 cc.).	
	Soluble ash (1.5395 gm.).	Insoluble ash (0.0047 gm.).	Total ash (1.5032 gm.).	Ash per 100 cc. fluid.	Total ash.	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.	gm.	gm.
Cl.....	0.7486	0	0.7486	0.3743	—	—
NaCl.....	—	—	1.2336	0.6168	—	—
SO ₃	0.0661	0.0027	0.0688	0.0344	0.0576	0.0288
S.....	—	—	0.0274	0.0137	—	0.0115
P ₂ O ₅	0.0042	0.0112	0.0154	0.0077	0.0130	0.0065
P.....	0.0018	0.0048	0.0066	0.0033	0.0056	0.0028
Na ₂ O.....	0.7729	0	0.7729	0.3864	—	—
Na.....	0.5737	0	0.5737	0.2868	—	—
K ₂ O.....	0.0444	0	0.0444	0.0222	—	—
K.....	0.0372	0	0.0372	0.0186	—	—
CaO.....	—	—	—	—	0.0321	0.0160
Ca.....	—	—	—	—	0.0229	0.0114
MgO.....	—	—	—	—	0.0019	0.0009
Mg.....	—	—	—	—	0.0012	0.0006

times. The filtrates were then combined, evaporated, after the addition of a little acetic acid, ashed, and analyzed. In the second part (B) the amount of sulfuric acid evidently was decreased, and the quantity of magnesium also a little diminished. From these facts we may conclude that a part of the sulfuric acid in the ash of amniotic fluid is produced from the sulfur of albumins.

TABLE IX.

	Amniotic fluid (200 cc.) (A).	Ash per 100 cc. fluid.	Amniotic fluid (200 cc.) (B).	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.
SO ₃	0.0518	0.0259	0.0227	0.0113
S.....	—	0.0103	—	0.0045
P ₂ O ₅	0.0142	0.0071	—	—
P.....	—	0.0030	—	—
CaO.....	0.0252	0.0126	0.0268	0.0134
Ca.....	—	0.0090	—	0.0096
MgO.....	0.0008	0.0004	0.0002	0.0001
Mg.....	—	0.0002	—	Trace.

To discover the relations of the phosphoric acid in the ash of amniotic fluid to albumins and other organic substances in the liquor, samples of the fluid from two women were divided into two parts. One part (A) was used for the quantitative determination of phosphoric acid. The other part (B) was freed from albumins by coagulation; the organic phosphorus substances were removed from the filtrate by extraction with ether, and the phosphoric acid was determined according to Neumann. The two results were nearly identical.

TABLE X.

	I. Amniotic fluid (80 cc.).		II. Amniotic fluid (100 cc.).	
	A (40 cc.).	B (40 cc.).	A (60 cc.).	B (100 cc.).
	Gm. per 100 cc. fluid.		Gm. per 100 cc. fluid.	
P ₂ O ₅	0.0049	0.0050	0.0045	0.0042
P.....	0.0021	0.0020	0.0019	0.0018

In order to decide whether calcium or magnesium combined with albumins, 100 cc. of amniotic fluid from two cases were divided into two equal parts; one part (A) was used for the quantitative determination of Ca and Mg; the other part (B) was freed from albumins with colloidal ferric hydroxide, and used for the same determination. The second half (B) was but little reduced in the quantity of Mg.

TABLE XI.

	Amniotic fluid (100 cc.).			
	A (50 cc.).		B (50 cc.).	
	gm.	Gm. per 100 cc. fluid.	gm.	Gm. per 100 cc. fluid.
CaO.....	0.0082	0.0164	0.0077	0.0154
Ca.....	0.0058	0.0116	0.0055	0.0110
MgO.....	0.0002	0.0004	Trace.	Trace.
Mg.....	0.0001	0.0002	"	"

To decide whether the amniotic fluid contains ether sulfuric acid, 100 cc. of the fluid were collected from four cases, the albumins were precipitated with colloidal ferric hydroxide, and filtered,

the whole volume being 1,000 cc. From the filtrate, 900 cc. (corresponding to 90 cc. of amniotic fluid) were evaporated to 85 cc.; 15 cc. of dilute hydrochloric acid and 15 cc. of 5 per cent barium chloride solution were added, and barium sulfate was filtered. The weight of barium sulfate produced here represents the inorganic sulfuric acid. The filtrate of barium sulfate was boiled on a water bath until the newly produced barium sulfate was entirely precipitated; then from its weight, the weight of ether sulfuric acid was calculated, as shown in Table XII.

TABLE XII.

	Amniotic fluid (100 cc.).	
		gm.
Inorganic sulfuric acid.....	SO ₃	0.0279
Ether " "	SO ₃	0.0006
Total " "	SO ₃	0.0285

In two experiments 30 cc. of amniotic fluid from two cases each, were reduced to ash, dissolved in hydrochloric acid, and the quantity of iron was determined by Neumann's method. The results are given in Table XIII.

TABLE XIII.

	Amniotic fluid (30 cc.).		Amniotic fluid (30 cc.).	
	gm.	Gm. per 100 cc. fluid.	gm.	Gm. per 100 cc. fluid.
Fe.....	0.00248	0.0082	0.00266	0.0088
Fe ₂ O ₃	0.00709	0.0236	0.0076	0.0253

Furthermore, in one case each of the 5th, 6th, 8th, and 9th months of pregnancy the quantity of sodium chloride was determined by Volhard's (12) method (Table XIV).

These values show that there is no marked difference to be found in the quantity of sodium chloride at different periods of pregnancy.

Summary.—(1) The amniotic fluid of women at the end of pregnancy always contains Cl, carbonic acid, sulfuric acid, phos-

TABLE XIV.

No. of amniotic fluid.	Cl	NaCl	Remarks.
	Gm. per 100 cc. fluid.		
118	0.3939	0.65	35th week of pregnancy.
123	0.3636	0.6	Middle of 8th month, still-born, weight of fetus, 1,800 gm., con- genital syphilis (?)
191	0.3211	0.53	5th month, artificial abortion for consumption.
227	0.3939	0.65	Beginning of 6th month.

phoric acid, Na, K, Ca, Mg, and Fe. (2) The amounts of Cl, phosphoric acid, K, Na, Ca, and Fe are nearly constant. (3) The greatest part of the inorganic salts is sodium chloride, which averages 75.2 per cent of the total ash. (4) A part of the sulfuric acid in the ash comes from the sulfur of albumins and a very small part comes from ether sulfuric acid. Therefore, the quantity of sulfuric acid in the ash depends on the quantity of albumins. (5) The average quantities of the inorganic components are shown in Table XV.

TABLE XV.

Amniotic fluid (100 cc.).			
Inorganic components.	Average gm.	Inorganic components.	Average gm.
Water.....	99.4880	P.....	0.0026
Solid substances.....	1.2425	Na ₂ O.....	0.3819
Organic substances.....	0.4504	Na.....	0.2834
Soluble ash.....	0.7690	K ₂ O.....	0.0232
Insoluble ash.....	0.0407	K.....	0.0194
Total ash.....	0.8098	CaO.....	0.0157
Cl.....	0.3695	Ca.....	0.0111
NaCl.....	0.6090	MgO.....	0.0005
SO ₃	0.0432	Mg.....	0.0003
S.....	0.0172	Fe ₂ O ₃	0.0244
P ₂ O ₅	0.0061	Fe.....	0.0085

d-Lactic Acid and Sugars in Human Amniotic Fluid.
d-Lactic Acid.

Zweifel (13) examined the urine of eclampsia patients and found an increase of ammonia and the existence of *d*-lactic acid. In the same cases he confirmed the existence of a certain quantity of *d*-lactic acid in the blood of maternal bodies, the blood of the umbilical cord, and the placenta. Ten Doeschate (14) also states that in eclampsia cases the *d*-lactic acid is always present in the blood and urine of maternal bodies, in the blood of the umbilical cord, and in the placenta. Because, however, in one case of normal confinement, a quantity of *d*-lactic acid was found in maternal blood corresponding to 0.029 per cent zinc lactate, in placenta corresponding to 0.119 per cent zinc lactate, Ten Doeschate concluded that Zweifel's assertion cannot be adequate, *i.e.*, the *d*-lactic acid is not the cause of eclampsia, but the convulsion in eclampsia provokes the accumulation of lactic acid. The relations between lactic acid in the blood and urine and obstetrical diseases were the subjects of the foregoing investigations; the presence of lactic acid in the amniotic fluid is examined for the first time in the following experiments.

Method.

Amniotic fluid was evaporated on a water bath to one-tenth its volume then poured into ten parts alcohol, and extracted. The alcoholic extract was concentrated, the residue dissolved in a small quantity of water, phosphoric acid added until the solution was strongly acid, and the solution extracted in a Suto extraction apparatus with ether for 24 hours. The brown residue from the ethereal extract was dissolved in water, treated with freshly prepared lead carbonate, heated on a water bath for nearly 30 minutes, and filtered. The filtrate was freed from lead by H_2S , the H_2S removed by vacuum distillation, zinc oxide added to the condensed solution, the mixture boiled on a water bath for 30 minutes, and filtered. The filtrate was decolorized by blood charcoal and the clear solution thus obtained was evaporated until the zinc lactate began to crystallize, when a little alcohol was added. As the white crystals of zinc lactate were not yet quite pure, they were washed several times with absolute alcohol, dissolved in a small quantity of hot water and brought again to crystallization.

It was then assured that the white crystals thus prepared were no other than those of zinc *d*-lactate by (1) examination of crystal shape, (2) Uffelmann's reaction, (3) Fletcher and Hopkins' reaction, (4) measurement of the specific rotation, (5) quantitative determination of the water of crystallization, (6) elementary analysis.

Experiment 1.—1,000 cc. of clean amniotic fluid collected from fourteen healthy women in labor, were treated as above, giving 0.9 gm. of zinc salt, colorless needles, or rhombic prisms, giving a positive Uffelmann's reaction and Fletcher and Hopkins' reaction.

$$[\alpha]_D^{20} = \frac{-0.4 \times 100}{2.506 \times 2} = -7.98^\circ.$$

0.3359 gm. salt lost 0.0433 gm. H_2O on drying at 110° to constant weight.

0.2045 " dry salt gave 0.0679 gm. ZnO .

0.0768 " " " " 0.0834 " CO_2 and 0.027 gm. H_2O .

Experiment 2.—1,000 cc. of yellowish green amniotic fluid collected from twenty cases and mixed with a small quantity of meconium, gave 1 gm. of zinc salt.

$$[\alpha]_D^{20} = \frac{-0.58 \times 100}{3.646 \times 2} = -7.95^\circ.$$

0.1567 gm. salt lost 0.0204 gm. H_2O .

0.211 " dry salt gave 0.0707 gm. ZnO .

Experiment 3.—2,000 cc. of amniotic fluid obtained from a case of acute hydramnios at the end of the 8th month of pregnancy, gave 1.917 gm. of zinc salt.

$$[\alpha]_D^{20} = \frac{-0.66 \times 100}{4.084 \times 2} = -8.08^\circ.$$

0.5143 gm. salt lost 0.0668 gm. H_2O .

0.112 " dry salt gave 0.0374 gm. ZnO .

0.2054 " " " " 0.073 gm. H_2O and 0.2187 CO_2 .

From the foregoing results it is possible to conclude that *d*-lactic acid is a constant component of human amniotic fluid.

TABLE XVI.

Zinc salt prepared from human amniotic fluid.				Zinc <i>d</i> -lactate.
Experiment.	1	2	3	
Crystals.	Needle-shaped or rhombic prisms.			
Specific rotation $[\alpha]_D^{20}$	-7.98°	-7.95°	-8.03°	-8.6°
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Water of crystallization	12.89	13.01	12.98	12.86
Zn.....	26.65	26.91	26.83	26.86
C.....	29.62	—	29.71	29.54
H.....	3.86	—	3.95	4.13

TABLE XVII.

Sugars.

Gürber and Grünbaum (16) found some fruit sugar in the amniotic fluid of cows, sheep, and swine, but could not find even a trace of sugars in the fluid of ten women examined in the same way. Grünbaum (17) states that the amniotic fluid of cats and dogs is free from fruit sugar. However, Ludwig (18) found 0.3 gm. of sugar in 4,000 cc. of amniotic fluid obtained by artificial puncture of fetal membranes of a diabetes patient, whose urine contained about 3.8 per cent sugar.

In order to decide whether human amniotic fluid is surely free from sugars, an aqueous solution, after being freed from lactic acid, was neutralized with calcium carbonate, and filtered. The voluminous precipitate of calcium phosphate was then mixed with water in a porcelain mortar and filtered again by suction. The filtrates were combined and concentrated to 100 cc. and used for the following examinations: Trommer's and Nylander's reactions were negative; the phenylhydrazine reaction was negative; and finally, the solution was optically inactive. The results, therefore, of these experiments confirm the statements of Gürber and Grünbaum that sugars are not a constant component of human amniotic fluid.

Summary.—(1) The *d*-lactic acid is a constant component of human amniotic fluid and amounts to 0.0726 per cent on an average. (2) Sugars are not to be found in human amniotic fluid.

Allantoin and Organic Bases in Human Amniotic Fluid.

According to Gusserow and Hermann, the urine of healthy bodies always contains a small quantity of allantoin, while the urine of pregnant women contains more. Wiechowski (19), however, states that he could find no allantoin in human urine, in 200 cc. of urine of a 1½ months old suckling baby, nor in the urine of a new-born child at its first day; furthermore, allantoin was absent in the amniotic fluid of two pregnant women. It is still an open question whether allantoin is a component of human amniotic fluid; the amino-acids or organic bases of human amniotic fluid have not been investigated.

In the present investigation, 3,064 cc. of amniotic fluid were precipitated with basic lead acetate and filtered. The filtrate was freed from lead, concentrated *in vacuo* at 50° to about 150 cc., and filtered. The filtrate was diluted with water to 200 cc., concentrated sulfuric acid added until the latter amounted to 5 per cent in volume, and the solution filtered. The filtrate was treated with 20 per cent phosphotungstic acid solution and, after standing for a night, filtered, giving a reddish white precipitate (A),

used to test for organic bases, and a filtrate (B), used to test for amino-acids and allantoin.

Organic Bases.—The precipitate (A) was washed several times with 5 per cent sulfuric acid, then mixed with barium hydroxide in a porcelain mortar, and diluted with water. Carbon dioxide was passed into this mixture, the barium carbonate filtered, and the filtrate distilled *in vacuo* to 50 cc.; addition of dilute nitric acid and silver nitrate gave a small quantity of brown precipitate (I), purine bases, and a filtrate (II).

Purine Bases.—Precipitate I gave a negative test for purine bases.

Hexone Bases and Choline.—Filtrate II was divided into two portions with silver nitrate and barium hydroxide; the resulting precipitate (III) was washed several times with dilute barium hydroxide and used for the preparation of histidine and arginine, while the filtrate (IV) was used to test for choline and lysine. Using the ordinary tests and methods of separation, histidine was demonstrated by a positive Pauly diazo reaction; a picrate was obtained as fine yellow, silken needles, where one would expect to find arginine, but its melting point was 182°. Lysine was isolated as the picrate, orange-yellow needles, melting at 252°. Choline was absent.

Allantoin.—Allantoin was isolated from filtrate B according to Wiechowski (20). (The filtrate from the mercury acetate was used to prove the presence of amino-acids.) The material obtained from the mercury acetate gave a positive Eppinger (21) glyoxylic acid reaction, and gave oxalic acid when heated with 15 per cent sodium hydroxide (Salkowski, 22). Thus, while pure crystals of allantoin could not be isolated, a very small quantity of allantoin probably is to be found in human amniotic fluid.

Amino-Acids.—The filtrate from the mercury acetate-allantoin precipitate was freed of Hg and found to contain 0.3 gm. N. The presence of amino-acids was tested for according to Ignatowski (23), but with negative results.

Summary.—The results of the experiment, which was performed with 3,000 cc. of amniotic fluid are as follows: (1) Neither purine bases, choline, nor monoamino-acids are to be found in amniotic fluid. (2) Histidine, lysine, and allantoin are all present in very small quantity. (3) The existence of arginine is still undecided.

Albumins, Urea, Uric Acid, Creatine, and Cholesterol in Human Amniotic Fluid.

Albumins.

Koettnitz (24) found propeptone in the amniotic fluid in two cases in pregnancy of 2½ and 2½ months, respectively; peptone and propeptone in four cases in the 10th month, and peptone

alone in one case in the 10th month; from these facts he concluded that the amniotic fluid is used as nourishment for the fetus at the beginning of pregnancy, and has the same use, though in much less degree, at the end of pregnancy. Weyl (25) confirmed the presence of mucin in the amniotic fluid of two women in hydramnios and determined the quantities as 0.1 and 0.2 per cent.

Experiment 1.—37 cc. of amniotic fluid were centrifuged, filtered by suction, treated with an equal volume of a saturated solution of ammonium sulfate, and filtered after standing over night. The filtrate heated on a water bath, gave a heavy precipitate; the filtrate of this precipitate gave no biuret reaction. The precipitate, obtained by the addition of ammonium sulfate solution, was washed several times with a half saturated solution of ammonium sulfate, until its filtrate gave no more albumin reactions; when dissolved in a small quantity of water and heated on a water bath at more than 70°, the solution became white and cloudy; when boiled and filtered, the transparent filtrate showed a very weak biuret reaction.

Experiment 2.—60 cc. of nearly clear and yellowish amniotic fluid were diluted with an equal volume of water and filtered by suction. When mixed with a few drops of acetic acid, the filtrate showed a white cloudiness, which could not be redissolved by the addition of excess acetic acid. After standing over night, the slight precipitate was filtered and washed with very dilute acetic acid. The precipitate, boiled with dilute sulfuric acid, gave a solution with positive Trommer's reaction. The filtrate of the precipitate produced by the addition of acetic acid was neutralized with sodium carbonate, and upon adding an equal volume of a saturated solution of ammonium sulfate, gave a cloudiness. The filtrate was heated on a water bath and filtered from the coagulated albumins, but this filtrate gave no sign of biuret reaction.

The precipitate produced by the addition of ammonium sulfate was washed with a half saturated solution of ammonium sulfate, dissolved in a small quantity of water and heated on a water bath at more than 70° when the solution became white and cloudy. When boiled and filtered, the transparent filtrate still showed a very weak biuret reaction.

Experiment 3.—According to Hohlweg and Meyer (26), a mixture of a volume of 1 per cent acetic acid with an equal volume of 5 per cent monopotassium phosphate solution was added to 80 cc. of amniotic fluid until it reacted acid against Congo red paper, but neutral against litmus paper; then an equal volume of a saturated sodium chloride solution was added; this mixture was boiled on a water bath to coagulate the albumins, and filtered. The filtrate obtained thus gave no biuret reaction.

In Experiment 2, the appearance of the glucoprotein, which was produced by the addition of acetic acid and was not soluble

in excess acetic acid, confirmed the existence of mucin in amniotic fluid; Experiment 1 demonstrated that albumin and a little globulin were present in amniotic fluid, but that no peptones were present. Experiment 3 also demonstrated that peptones were absent. But whether the very weak biuret reaction in the filtrate of the coagulable albumins in Experiments 1 to 3 was due to the presence of albumoses could not be determined.

Quantitative Determination of Coagulable Albumins.

Prochownick (1) found 0.71 to 0.06 per cent coagulable albumins in the amniotic fluid of eleven women; Ahlfeld (27) states that the amniotic fluid of seven out of twenty women contained 25 to 50 volume per cent albumins, and from this fact he argued that amniotic fluid must be a nourishment for the fetus; however, his argument is not adequate as the following results of other authors indicate. According to Schroeder (2), the amount of albumins in the amniotic fluid stands in a close relation to the quantity of hemoglobin in the maternal blood; in 50 cases it amounted to 0.03 to 0.308 per cent. In the mature fetus, it averaged 0.1681 per cent, and 0.1578 per cent in the immature fetus. In Döderlein's (6) experiments with fifteen cows, the quantity of albumins in the amniotic fluid amounted to 0.042 to 0.455 per cent; it increased gradually as the pregnancy proceeded. Jacqué found 0.023 to 0.058 per cent albumins in the amniotic fluid of the sheep fetus, when less than 14 cm. in length, and 0.1 per cent albumins when over 30 cm. in length.

In the present experiments, human amniotic fluid was first filtered through fourfold gauze, then centrifuged, and filtered by suction. Thus prepared, the fluid was diluted with three parts of water, sodium chloride added to make 1 per cent, the solution made weakly acid with 1 per cent acetic acid, and boiled on a water bath. This completely coagulated the albumins which were filtered through a tared ashless filter, washed with warm water, then with alcohol, and finally with ether. Thus treated, the albumins were dried on the filter at 110° and weighed. Since the coagulated albumins thus obtained usually contained more or less ash, this ash was determined, and from it the weight of pure coagulable albumins calculated. The value obtained by the above method is the sum of the albumin, globulin, and mucin, but this may be considered coagulable albumins in a narrow sense, since the quantity of mucin in the amniotic fluid is very small.

The quantity of the coagulable albumins in the amniotic fluid of fifteen women at the end of pregnancy varied from 0.092 to

0.421 per cent, with an average of 0.226 per cent. In two cases of the 5th and 6th months of pregnancy, respectively, the amount of coagulable albumins is not markedly different from the amount at the end of pregnancy. The results of these experiments are shown in Table XVIII.

TABLE XVIII.

No. of experiment.	No. of amniotic fluid.	Quantity of amniotic fluid.	Coagulable albumin.		Remarks.
				Gm. per 100 cc. fluid.	
		cc.	gm.		
1	83	10	0.0421	0.421	
2	85	5	0.0149	0.299	
3	86	10	0.0160	0.160	
4	87	10	0.0229	0.229	
5	89	10	0.0277	0.277	
6	90	10	0.0259	0.259	
7	91	10	0.0149	0.149	
8	92	5	0.0046	0.092	
9	95	10	0.0215	0.215	
10	96	10	0.0352	0.352	
11	97	10	0.0115	0.115	
12	104	10	0.0131	0.131	
13	106	10	0.0190	0.190	
14	114	10	0.0265	0.265	
15	115	10	0.0238	0.238	
Average.				0.226	
16	191	10	0.0384	0.384	5th month, artificial abortion for consumption.
17	227	10	0.0416	0.416	Beginning of 6th month.

Quantitative Determination of Urea and Distribution of Nitrogen.

Although Scherer, Mack, and Calberg denied the existence of urea in the amniotic fluid, Woeler discovered it by exact investigation. Later, other investigators have given the following values: Funke, 0.38 per cent; Letzmann, 0.05 per cent; Majewski, 0.34 to 0.42 per cent; Beale (28), 0.35 per cent; Siewert (29), 0.0352 per cent; Winckel (30), 0.086 to 0.42 per cent; Gusserow (31) 0.14 to 0.35 per cent. Prochownick (7) determined the quantity of urea as nitric urea in two cases in the first half of pregnancy and also in eleven cases at the end of pregnancy, and found 0.0155 to 0.034 per cent; since the amount of urea was small at the beginning and greater at the end

of pregnancy, he concluded that urea in the amniotic fluid is secreted from the skin and the kidneys of the fetus, and that the fluid itself is a product of the fetus. Schöndorff (32) stated that the amount of urea in the amniotic fluid (0.0611 per cent) is about the same as that in the blood and the milk.

In the present investigations, the ammonia was determined by the method of Brugsch and Schittenhelm (33); Marshall's (34) method was used for the determination of urea, and Kjeldahl's for the total nitrogen. The albumin N was determined in the precipitate obtained with colloidal ferric hydroxide. The amount of ammonia varied from 0.0051 to 0.0013 per cent, with

TABLE XIX.

No. of experiment.	No. of Amniotic fluid.	Ammonia.	Urea.	Total N.	Albumin N.	Rest N.	Ammonia N.*	Urea N.	Remaining N.	Remarks.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1	112	0.0040	0.0276							Mixed with meconium.
2	113	0.0023	0.0570							
3	115	0.0015	0.0519							
4	117	0.0023	0.0666							Mixed with meconium.
5	122	0.0017	0.0504							
6	132	0.0013	0.0297							
7	143	0.0044	0.0204							Twins.
8	211	0.0028								
9	219	0.0051								
10	227	0.0017	0.0195	0.1036	0.0868	0.0168	0.0014	0.0091	0.0063	
11	232	0.0034	0.0300							
12	237	0.0051	0.0180	0.0588	0.0441	0.0147	0.0042	0.0084	0.0021	
13	243	0.0025	0.0195	0.0518	0.0336	0.0182	0.0021	0.0091	0.0070	
14	247	0.0034	0.0255							
15	248	0.0029	0.0187	0.0770	0.0635	0.0135	0.0024	0.0087	0.0024	
16	250	0.0029	0.0174	0.0840	0.0672	0.0165	0.0024	0.0061	0.0083	
Average.		0.0029	0.0323	0.0750	0.0590	0.0159	0.0025	0.0083	0.0052	

* The ammonia N corresponds to 15.7 per cent of the so called rest N, and the urea N corresponds to 52.2 per cent of the rest N.

an average of 0.0029 per cent; the urea varied from 0.0666 to 0.0174 per cent, with an average of 0.0323 per cent. Five experiments on the distribution of N gave average values of: albumin N, 0.059 per cent; rest N, 0.0159 per cent; ammonia N, 0.0025 per cent; urea N, 0.0083 per cent. These results are seen in Table XIX.

Uric Acid.

653 cc. of amniotic fluid were freed from coagulable albumins by boiling with dilute acetic acid, and then by adding 5 per cent monopotassium phosphate solution and boiling. The filtrate was then tested for uric acid by Schroeder's (35) method. The resulting silver compound was freed from silver, the residue extracted with a dilute sodium carbonate solution, the solution treated with acetic acid, and evaporated. After a day, some amorphous reddish sediment separated; this was insoluble in cold water, but easily dissolved upon warming and separated again upon cooling. A part of this sediment gave a marked murexide reaction, but there was not enough for an elementary analysis. A second experiment gave negative results. Uric acid in small quantity is contained in the amniotic fluid during confinement.

Creatine and Creatinine.

Gönner (36) states that creatine and creatinine should be contained in the amniotic fluid, but that they have not been secured experimentally.

In one experiment, the filtrate from the uric acid precipitate (silver and magnesia) was freed from silver by hydrochloric and nitric acids and used for the preparation of creatine according to Neubauer and Salkowski's (37) method, with negative results, neither creatine nor creatinine being found. In another experiment, 420 cc. of pure amniotic fluid, treated in the same way, showed the presence of creatine but creatinine was absent. Amniotic fluid during confinement contains creatine in very small quantity, but no creatinine.

Hippuric Acid.

Until the present experiments, no investigation has been made for hippuric acid as a component of the amniotic fluid.

526 cc. of amniotic fluid were precipitated with lead acetate, the lead in the filtrate was removed with H_2S , the filtrate concentrated *in vacuo* to a syrup, and extracted with alcohol; the residue was dissolved in a small quantity of hot water, acidified with dilute hydrochloric acid and extracted with acetic ether. This extract was washed with water, and, upon evaporation, gave some brown residue which was extracted with petroleum ether. The insoluble part contained no hippuric acid; two other similar experiments were likewise negative. Hippuric acid is not a component of human amniotic fluid.

Cholesterol.

Gönner (36) states that the amniotic fluid contains cholesterol, but no experimental investigation of it could be found in the literature.

For these experiments, 905 cc. of amniotic fluid were extracted with ether in a Suto extraction apparatus for 48 hours, the extract was saponified with dilute alcoholic potassium hydroxide, the residue treated with 20 cc. of hot water, and after cooling, extracted with ether three times. The residue was extracted with petroleum ether, and the soluble portion dissolved in a mixture of ether and alcohol, and the solution allowed to evaporate spontaneously. White, mica-like, glossy, squamiform crystals were obtained; examined under a microscope, they were colorless, very thin, rhombic or irregularly square tablets, sometimes broken at a corner, and piled closely one on top of another. They melted at 146° , were optically active, and gave positive Salkowski's, Liebermann and Burchard's, and Schiff's reactions. From this, it is evident that the substance obtained by extraction with ether from human amniotic fluid is cholesterol, and the cholesterol is beyond a doubt a component of human amniotic fluid.

Summary:—(1) The human amniotic fluid at the end of pregnancy always contains coagulable albumins (an average of 0.226 per cent, inclusive of mucin); albumin is its greatest constituent, but globulin is present in traces; the amount of albumins in the amniotic fluid in the first half of pregnancy is not remarkably different from that at the end. (2) Mucin is also contained, but in quantity too small to determine; peptone and albumoses are

not found. (3) Ammonia and urea are constant components; the amount of ammonia is, on an average, 0.0029 per cent; the amount of urea, on an average, is 0.0323 per cent, and both together furnish about 70 per cent of the so called rest N. (4) Uric acid and creatine, in very small amounts, are present, but neither creatinine nor hippuric acid is found. (5) Cholesterol is a component of human amniotic fluid.

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UREA FORMATION BY THE PLACENTA.

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INTRODUCTION.

The formation of urea is apparently a function of all tissues. A great deal of investigation has been carried on in an attempt to determine whether or not any particular tissue or groups of tissues possess this ability in demonstrable form, with the result that no specific urea-forming power has been found for any. The careful work of Hoagland and Mansfield¹ on muscle tissue was not productive of results favorable to the view that the formation of urea is a special property of this tissue. Nor could Fiske and Sumner² localize its production in liver or muscle. It would, on the surface, seem useless to attempt any study of this question with the placenta, were it not for the fact that the various methods used have produced conflicting results, and on account of the unique position of the placenta in the metabolic interchange between mother and fetus. Since the synthetic power of the placenta in so far as glycogen is concerned has been indicated by Driessen³ and others, there is no reason to suppose that in common with other tissues it cannot form urea. As with other tissues, on autolysis leucine and tyrosine are formed by the placenta,⁴ but in the literature on the placenta no mention is made of the occurrence of urea.

In view of these facts and in order to make an attempt to determine whether the placenta forms urea in determinable amounts, experiments were conducted to this end, utilizing the procedure

¹ Hoagland, R., and Mansfield, C. M., *J. Biol. Chem.*, 1917, xxxi, 487.

² Fiske, C. H., and Sumner, J. B., *J. Biol. Chem.*, 1914, xviii, 285.

³ Driessen, L. F., *Arch. Gynäk.*, 1907, lxxxii, 278.

⁴ Mathes, P., *Centr. Gynäk.*, 1901, xxv, 1385.

hereinafter described, which was arrived at after various unsuccessful experiments along the lines of other workers.

The following is a brief report of one phase of the work. The original idea was to make an exhaustive study of urea formation in placentas from normal and toxemic pregnancies, with a view not only to ascertain the normal course of the processes, but also to determine if possible the seat of the distressing toxemias often accompanying pregnancy. Unfortunately the war has interrupted this line of work and has prohibited a more complete survey and discussion of the problems in this paper.

Procedure.

The fresh placentas were prepared for analysis and the ammonia and urea determined as described in a previous publication.⁵ Masses of the pulped tissue were then put into weighed bottles and the amounts of tissue ascertained by the increments in weight of containers and contents, usually about 150 gm. An equal weight of distilled water containing 5 cc. of a mixture of equal parts of chloroform and toluene was then added and the whole well mixed by shaking. Bits of pulp adhering to stoppers and sides of bottles were washed down with a few cc. of the diluent kept out for the purpose. The containers and contents were then put in the cold room or left in the laboratory as the occasion demanded.

Each day, after shaking the suspended pulp, the contents of the bottles were thrown onto a large funnel containing a small plug of cotton, and 10 cc. portions of the resulting filtrate were used for the analyses. The residuum in the funnel was then carefully returned to the bottles together with the washings, account being kept of the weights of added and removed fluid. In this way the removal of a fair proportion of the products of autolysis allowed the processes to continue unhindered by their accumulation.

All calculations were made to a basis of 100 gm. of fresh tissue. The results of the analyses, together with the per cent change from the initial quantities found, are recorded in Tables I to IV.

⁵ Hammett, F. S., *J. Biol. Chem.*, 1918, xxxiii, 381.

TABLE I.
Urea Formation at 4°C. in Placentas from Normal Pregnancies.

Case No.	39			40			41			42			47			48			49			24			36			37		
	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.	Urea N.		Increase.			
	mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent		mg.	per cent				
1	4.7	—	9.1	—	8.3	—	6.0	—	11.1	—	7.4	—	9.2	—	4.8	—	8.3	—	6.9	—	—	—	—	—	—	—	—			
2	9.2	96	7.7	—	15	31	6.3	—	11.6	4	6.0	—	7.8	—	5.6	17	8.3	0	6.8	—	—	—	—	—	—	—				
3	—	—	8.8	—	3	9.9	19	7.0	6	—	7.5	2	—	—	5.3	11	8.1	—	6.8	—	—	—	—	—	—	—				
4	6.0	28	7.7	—	15	—	7.7	17	12.1	8	7.6	3	9.3	1	5.7	19	8.1	—	7.3	5	—	—	—	—	—	—				
5	9.6	104	9.0	—	1	12.5	50	8.1	23	10	7.9	7	10.6	16	5.8	20	—	—	—	—	—	—	—	—	—	—				
6	10.3	118	9.2	1	12.6	52	—	—	12.5	13	—	—	—	11	—	—	—	—	—	—	—	—	—	—	—	—				
7	9.6	104	9.9	9	12.9	55	8.8	34	13.1	18	7.6	3	11.5	25	7.9	66	9.0	8	6.0	—	—	—	—	—	—	—				

TABLE II.
Urea Formation at 16°C. in Placentas from Normal Pregnancies.

Case No.	Day.	39		40		41		42		47		48		49		50		51		52	
		Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
		mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1		4.7	—	9.1	—	8.3	—	6.6	—	11.1	—	7.4	—	9.2	—	8.5	—	7.7	—	12.9	—
2		10.9	132	13.4	47	11.3	36	7.6	15	14.5	31	8.4	13	10.0	8	12.9	52	9.1	18	13.4	4
3		—	—	11.9	30	13.3	61	10.1	53	—	—	9.2	24	—	—	13.2	55	11.2	45	17.7	37
4		10.5	122	19.8	118	—	—	11.2	70	16.4	48	10.5	42	12.6	37	13.3	57	11.9	55	17.0	31
5		13.0	176	22.8	150	14.7	77	13.1	99	17.3	56	11.8	59	13.9	51	17.1	100	14.4	87	18.9	47
6		11.9	154	24.2	168	15.8	91	—	—	18.6	68	—	—	15.2	65	18.0	111	14.0	81	19.7	53
7		14.1	201	26.1	187	15.4	86	13.1	99	17.9	61	11.9	61	15.2	65	19.0	122	13.9	80	21.0	62

TABLE III.
Urea Formation at 4°C. in Placentas from Toxemic Pregnancies.

Case No.	Day.	46		53		25		26		27		29		30		31		32		33	
		Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
		mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1		24.4	—	10.2	—	5.8	—	4.4	—	15.7	—	5.6	—	4.9	—	2.5	—	14.6	—	5.3	—
2		—	—	11.6	14	6.2	7	6.4	45	—	—	8.4	50	5.3	8	7.3	192	13.9	—	—	—
3		28.4	16	12.1	19	6.9	19	8.5	94	15.0	—4	10.9	94	8.0	65	8.1	223	14.9	2	6.6	25
4		34.3	41	12.5	22	—	—	—	—	16.9	8	12.6	126	9.2	88	9.5	279	14.1	—4	8.3	56
5		34.8	43	—	—	7.1	22	9.0	104	13.1	—17	—	—	—	—	—	—	—	—	10.1	91
6		34.3	41	12.4	21	8.3	42	9.0	104	17.2	11	12.9	131	8.7	78	7.7	207	15.6	7	11.0	104
7		36.3	49	12.6	24	7.8	34	5.2	18	19.5	24	13.2	136	9.1	86	8.2	228	17.2	18	11.8	122

TABLE IV.
Urea Formation at 16°C. in Placentas from Toxemic Pregnancies.

Case No.	Day.	46		53		38		43		44		45		54		55		56	
		Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent	Urea N.	Increase per cent
1		24.4	—	10.2	—	9.0	—	4.7	—	8.2	—	7.2	—	7.6	—	15.1	—	10.0	—
2		—	—	13.1	28	12.4	38	4.0	—15	13.2	61	9.7	35	9.9	30	15.1	0	10.0	0
3		20.0	10	14.4	42	—	—	8.6	83	15.3	87	—	—	12.8	69	14.9	—2	14.7	47
4		35.5	45	15.2	49	15.7	75	—	—	—	—	12.7	76	13.4	76	15.7	4	14.8	48
5		37.3	53	—	—	10.5	17	12.8	173	20.5	151	13.9	93	14.7	94	—	—	—	—
6		38.1	56	16.4	61	12.9	43	12.7	170	18.8	128	14.0	94	—	—	14.7	—3	15.9	59
7		38.8	59	16.9	66	12.7	41	12.7	170	19.3	135	15.1	110	17.2	126	14.4	—5	16.4	64

Results.

An inspection of Tables I to IV at once reveals the fact that urea, as determined by the soy bean urease, increases in amount from day to day in watery suspension of pulped placenta tissue kept in glass bottles and in the presence of chloroform and toluene.

As would be expected, an increase in the temperature at which the tissues were kept resulted in an increase in the amounts of urea formed in a given period. This is plainly shown by a comparison at the two temperatures of Cases 39, 40, 41, 42, 47, 48, and 49 in Tables I and II, of placentas from normal pregnancies; and a like comparison in Cases 46 and 53, Tables III and IV, of placentas from toxemic pregnancies.

An insufficient number of correlated cases from toxemic pregnancies prevents any conclusion as to the relativity of the action involved.

DISCUSSION.

It is evident that the placenta can form urea under certain circumstances. The processes leading up to this product are in question. It may be simply a splitting of the urea residue from arginase, or it may be a heightening to determinability of the usual processes taking place in extrauterine life, whose course from deaminization, through ammonium carbamate, to urea, is fairly well established.

Whatever the source of the urea found to be formed by placenta tissue, these results give evidence that this organ as well as being excretory in function, is also actively concerned in the preparation of the nitrogenous by-products of the fetus for excretion.

It is our present idea that the fetus is only faintly able if at all, to perform for, of, and by itself the functions of preparing its nourishment in assimilable form, or of breaking down its by-products into compounds ready for excretion; and that the placenta is interposed between mother and fetus to remove as far as possible these extra burdens from the maternal organism. These questions can obviously be settled only by continuation of the study of the biochemistry of placenta and fetal tissues.

CONCLUSION.

Urea formation is one of the properties of the placenta that can be conclusively demonstrated, although the processes leading up to its production can only be surmised. This finding lends further importance to the placenta as an intermediary organ between mother and fetus for mutual protection, as well as for the carrying on of processes undeveloped by the fetus, yet necessary for its continuance.

THE MAXIMUM PRODUCTION OF GLUTAMINE BY THE HUMAN BODY AS MEASURED BY THE OUTPUT OF PHENYLACETYLGLUTAMINE.

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Phenylacetic acid when fed to animals such as horses (1), dogs, rabbits (2), and sheep (3), is excreted in the urine as phenaceturic acid. This combination of phenylacetic acid with glycoll, however, does not take place in the human body. In man the phenylacetic acid combines with glutamine and is excreted in the urine as phenylacetylglutamine (4). E. Salkowski and H. Salkowski (2) find after feeding phenylacetic acid to rabbits an increase of almost 75 per cent in the total SO_3 output, but at the same time a decrease in the amount of "combined SO_3 ." The increase in the amount of inorganic sulfates in the urine they attribute to an increased catabolism of protein material necessary for the preparation of sufficient amount of glycoll to detoxicate the phenylacetic acid. The decrease in ethereal sulfates they believed to be due to the action of phenylacetic acid as a disinfecting agent with a subsequent decrease in the amount of intestinal putrefaction.

As glutamine (5) rather than glutamic acid seems to be the primary amino-acid constituent of protein, we thought it important to determine the maximum amount of this acid which the human body is able to furnish for the detoxication of phenylacetic acid. We wished also to know whether the ingestion of phenylacetic acid would be followed by increased sulfur metabolism in the human body as reported by previous investigators (2) in animals. Lastly, we wished to determine the effect of the acid in the course of intestinal putrefaction as measured by the excretion of urinary indican, as well as by the amount of ethereal sulfates excreted in the urine.

EXPERIMENTAL.

The subject was a man weighing 60.2 kilos. He was maintained throughout the entire experimental period on a uniform diet. The experiment covered a period of 24 days. On the 5th day of this period 2.5 gm. of phenylacetic acid were ingested, on the 7th day 5 gm., on the 10th day 7.5 gm., on the 13th day 10 gm., and on the 21st day 15 gm. The acid in each case was ingested as a solution of the sodium salt. The entire amount of the solution was taken at once, covering a period of not more than 3 to 5 minutes. The urine was collected in 24 hour periods, beginning at the time of the ingestion of the acid. The urine volume was first measured, and sufficient amount taken for the indican and sulfate determinations on that day and the day following the acid ingestion; the remaining urine volume was measured, exactly neutralized with sodium carbonate, and evaporated on the water bath to a thick syrup. The determination of the indican was made in duplicate upon each urine sample by the method of Ellinger (6). Each cc. of the Wang solution (7) employed in the titration was found upon standardization to be equivalent to 0.300 mg. of indigo,¹ or 0.576 mg. of indican. The ethereal sulfates and total sulfates in the urine were determined by the method of Folin (8). To determine the quantity of phenylacetylglutamine, the evaporated urine was made acid with phosphoric acid (Congo red as indicator) and extracted in a continuous extracting apparatus with absolute ethyl acetate. The amount of ethyl acetate used during each extraction was about 300 cc., and the time of extraction varied between 1 and 2 hours. The extracting was continued until the ethyl acetate was found on evaporation to contain no more phenylacetylglutamine. To obtain the phenylacetylglutamine the ethyl acetate extracts were placed on ice for 24 hours. At the end of this time, the white flaky crystals of the substance appeared on the sides and bottom of the flask. These crystals were scraped loose and filtered off, the mother liquor was evaporated to one-half the volume, and again placed in the ice box for 24 hours. This process was repeated until all the compound was crystallized out of the solution. The

¹ The indigo used in standardizing the Wang solution was furnished by Professor P. B. Hawk of Jefferson Medical College.

phenylacetylglutamine from the different extractions was united and recrystallized from absolute ethyl acetate, dried, and weighed. In each extract there occurred about an equal amount of phenylacetylglutamine and phenylacetylglutamine urea. In order to split off the urea and convert the urea compound into phenylacetylglutamine, a water solution of the urea compound was made slightly alkaline with barium hydroxide, the solution treated with carbon dioxide to remove the excess of barium, filtered, and the filtrate evaporated to dryness at a low temperature on the water bath. The residue, consisting of the barium salt of phenylacetylglutamine and urea, was extracted several times with hot absolute alcohol to remove the urea. The insoluble barium salt was separated from the urea by filtration. The barium salt of the phenylacetylglutamine was dissolved in a small amount of water, made acid with phosphoric acid, the barium sulfate filtered off, and the phenylacetylglutamine extracted from the concentrated solution with ethyl acetate.

DISCUSSION.

The results of the experiment are summed up in Table I.

On account of the toxicity of the phenylacetic acid, it was impossible for the subject to ingest more than 15 gm. of the acid. Even 5 gm. caused thirst, a slight feeling of dizziness, and nausea. The 10 gm. dose seemed to produce no exaggeration of these symptoms, but after ingestion of 15 gm. the subject noticed marked signs of poisoning, not unlike those following the ingestion of large quantities of alcohol. The daily urine volume which generally measured 800 to 900 cc. rose to at least 1,000 cc., and in one instance to 1,300 cc. after the ingestion of more than 5 gm. of phenylacetic acid. The increase in sulfur metabolism noted by Salkowski and Salkowski (2) after feeding the same acid to rabbits is not borne out in this experiment.³ On the 13th day of the experiment after an ingestion of 10 gm. of the acid, there was a rise in the total SO_4 output from 1.6862 gm. on the previous day to 2.5107 gm. This, although a marked rise, is even lower than the amount excreted the 3rd day before the ingestion of any of the acid.

³ The effect of phenylacetylglutamine production on nitrogen metabolism is being studied and will be reported later.

TABLE I.

Day of experiment.	24 hr. urine, vol- cc.	Phenylacetic acid ingested.	Phenylacetylgluta- mine recovered from urine.	Phenylacetic acid conjugated with glutamine.	Phenylacetic acid recovered from urine as phenyl- acetylglutamine.	Glutamine extract- ed from urine as phenylacetylglu- tamine.	Total SO ₂ excreted in urine during 24 hrs.	Inorganic SO ₂ excret- ed in urine dur- ing 24 hrs.	Ethereal SO ₂ excret- ed in urine dur- ing 24 hrs.	Indican excreted in urine during 24 hrs.	Relation of eth- ereal SO ₂ to total SO ₂ for 24 hr. pe- riod.	Relation of indican SO ₂ to ethereal SO ₂ .
1	860						1.8049	1.5905	214.4	23.82	1: 8.41	1: 19.96
2	830						1.8467	1.6843	162.4	27.26	1: 11.37	1: 13.21
3	990						2.6013	2.3340	267.3	26.44	1: 9.73	1: 22.42
4	920						2.5340	2.3518	182.2	22.86	1: 13.41	1: 17.67
5	900	2.5	2.02	1.1332	52.88	1.1070	2.1040	1.9683	135.7	12.90	1: 15.48	1: 23.33
6	1,010						2.7792	2.5702	209.0	28.16	1: 13.31	1: 16.46
7	1,100	5.0	6.16	3.3820	67.67	3.3264	2.7401	2.5848	164.3	00.00	1: 16.73	1: 00.00
8	970						1.9050	1.6972	207.8	00.00	1: 9.16	1: 00.00
9	820						2.3440	2.1473	196.7	12.66	1: 11.91	1: 34.44
10	1,070	7.5	7.23	3.7241	49.65	3.6873	2.4844	2.3516	132.8	14.28	1: 14.86	1: 20.63
11	820						2.0066	1.9471	159.5	9.76	1: 12.54	1: 36.24
12	785						1.6862	1.5314	154.8	5.39	1: 10.89	1: 63.64
13	950	10.0	10.07	5.1870	51.87	5.1357	2.5107	2.3237	167.0	31.89	1: 15.03	1: 12.00
14	840						1.6403	1.4835	156.8	27.32	1: 10.46	1: 12.76
15	700						1.8446	1.6903	154.3	28.11	1: 11.95	1: 12.18
16	900						1.7648	1.6272	137.6	29.02	1: 12.82	1: 10.53
17	1,200						1.6680	1.4850	183.0	30.24	1: 9.11	1: 13.42
18	690						1.5998	1.4688	131.0	19.57	1: 12.21	1: 14.84
19	815						1.7077	1.5573	150.4	22.61	1: 11.35	1: 14.75
20	820						2.3319	2.0873	244.6	26.59	1: 9.53	1: 20.40
21	1,305	15.0	14.75	7.5077	50.65	7.5225	2.0398	1.9034	105.5	22.39	1: 10.31	1: 10.46
22	860						2.2621	2.0654	196.7	40.87	1: 11.49	1: 10.65
23	780						1.9707	1.8060	164.9	6.27	1: 11.95	1: 58.30
24	720						1.8294	1.6830	146.4	8.70	1: 12.50	1: 37.32

On the 10th day, after receiving 7.5 gm. of the acid, there was an increase in total SO_4 of 0.1404 gm. or 5.95 per cent over the previous day. In all other cases there is even a decrease in the total amount of SO_4 excreted in the urine. There is no marked decrease in the amount of ethereal sulfates after the ingestion of the acid except on the 21st day of the experiment, when the amount fell to less than half the amount of the previous day and was approximately one-half the amount excreted on the succeeding day. There was also a relative decrease in ethereal sulfates in every case as is shown by the proportion of total SO_4 to ethereal SO_4 .

The influence of the phenylacetic acid on putrefaction in the intestine is best measured by the indican (9) output in the urine, as the amount in each case is affected by the ingestion of the acid. The influence of the phenylacetic acid on the indican excretion is most marked on the 1st or 2nd day after the ingestion of the acid. After a 5 gm. dose of the acid, the indican entirely disappeared for the same day and the day following, while on the 13th day 10 gm. of the acid caused only a slight decrease in the amount of indican excreted. After the ingestion of 15 gm., there is a very slight drop in the 24 hour indican value followed the next day by a marked increase. The decided lowering of the indican content of the urine first appeared on the 2nd and 3rd days after acid was taken. The ratio of indican SO_4 to total ethereal SO_4 shows that the production of indican was retarded to a much greater extent than the other compounds constituting the remainder of the ethereal sulfates.

In every case the amount of glutamine furnished by the body for the detoxication of the phenylacetic acid was approximately one-half the necessary amount. Regardless of the size of the dose of acid, only about 50 per cent of the acid appeared in the urine as phenylacetylglutamine.

After a dose of 2.5 gm., 2.02 gm. of the compound, corresponding to 52.88 per cent of the acid ingested, were isolated from the urine. After ingesting 15 gm. of the acid, 14.75 gm. of phenylacetylglutamine were recovered from the urine. In this case 50.65 per cent of the acid ingested was recovered. An exception was found after a 5 gm. dose, when the amount of acid regained amounted to 67.67 per cent with a collection of 3.3820 gm. of the

compound. The amount of glutamine furnished in each case was proportional to the amount of phenylacetic acid ingested regardless of the size of the dose. The largest quantity of glutamine isolated from the urine in combination with the phenylacetic acid was 7.5225 gm. after a dose of 15 gm. of the acid. It is probable that more of the phenylacetylglutamine would have appeared in the urine after each dose of the acid, had the acid been ingested at regular intervals covering a period of 10 or 12 hours. There may have been a reversible reaction at work thus decreasing the amount of the phenylacetic acid compound in the urine, or the remainder of the acid yet unaccounted for may have been detoxicated and eliminated through the urine as an entirely different compound.

CONCLUSION.

1. No marked increase in sulfur metabolism followed the ingestion of phenylacetic acid by man, as is shown by the very slight increase in the total SO_4 content of the urine. This does not agree with the findings of the experiment on rabbits by Salkowski and Salkowski.

2. Intestinal putrefaction is decreased after ingesting phenylacetic acid. The amount of urinary indican was decreased as well as the total ethereal sulfates.

3. About 50 per cent of the phenylacetic acid ingested was detoxicated in each case by uniting with glutamine. The largest amount of glutamine excreted was 7.5225 gm. after a dose of 15 gm. of phenylacetic acid. The amount of phenylacetylglutamine isolated from the urine after the 15 gm. dose of the acid was 14.75 gm.

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BEHAVIOR OF SUDAN III IN THE ANIMAL ORGANISM.

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The fat-soluble dye Sudan III, since its first preparation by Nietzki¹ in 1880, has been used extensively in the study of problems directly and indirectly associated with vital staining and the metabolism of fat. The work of Daddi, Gage, Riddle, Mendel, Whitehead, and many others, including that recently published by Hatai,² has shown how fat can be traced in the body by means of oil-soluble dyes.

In their paper on the behavior of fat-soluble dyes in the animal organism, Mendel and Daniels³ discussed the toxicity, absorption, transport, and excretion of this dye. They stated that it is apparently non-toxic, inasmuch as animals fed over long periods of time showed no deleterious results. When fed in solution in fat it may enter the organism from the alimentary tract through the lymphatics or by the portal circulation dissolved in reabsorbed bile, a cycle becoming established between the intestine, bile, and blood. The dye is eliminated in the feces alone, except when an alimentary lipuria arises; then it may occur in the urine. Moreover, it is deposited in the adipose tissues and bone marrow, stained fat being no less available to the organism than unstained.

These findings were later discussed by Salant and Bengis⁴ in their physiological and pharmacological studies of fat-soluble dyes. Using exceedingly large doses, they found that Sudan III was not pronouncedly toxic. Administered intraperitoneally, subcutaneously, intravenously, or by mouth the dye was reported

¹ Nietzki, R., *Ber. chem. Ges.*, 1880, xiii, 800.

² Hatai, S., *J. Exp. Zool.*, 1918, xxvi, 101.

³ Mendel, L. B., and Daniels, A. L., *J. Biol. Chem.*, 1912-13, xiii, 71.

⁴ Salant, W., and Bengis, R., *J. Biol. Chem.*, 1916, xxvii, 403.

to be excreted by the kidney, sometimes found in the bile, and deposited in the adipose tissue only. When observed a few hours after experiment, the central nervous system sometimes showed faint traces of color. Salant and Bengis contended that the absence of dye in the urine as reported by Mendel and Daniels³ was due to the abnormality of the animals, because only in the case of diseased animals did Salant and Bengis find absence of dye in the urine. This outcome they ascribe to decreased permeability of the kidneys.

The recent work of Hatai² on the several effects of feeding Sudan III to rats may well be subjected to criticism and maybe to revision in the light of the results reported below. He found that the feeding of Sudan III to young rats retarded the growth of the thymus, testes, and ovaries. Postmortem examination showed no trace of dye in the brain, spleen, or heart; but its presence was noted in the liver, pancreas, and kidney.

The original purpose of the present experiments was to reinvestigate the seemingly contradictory statements with regard to the path of elimination of the fat-soluble dye Sudan III, and particularly the contention of Salant and Bengis regarding the excretion of Sudan III by the kidneys. The products of various manufacturers were used and gave varying results, which led to interesting observations on the doubtful purity of preparations of Sudan III obtainable on the market at present. Experiments made with a purified product, on the other hand, gave definite results, thus showing the need for careful examination of all preparations of Sudan III used in scientific work to guarantee its purity before drawing conclusions supposedly based upon its properties as a pure, neutral fat-soluble dye.

Properties of Sudan III.

There are many references in the literature to the preparation and properties of Sudan III, which may be briefly summarized as follows:

Sudan III, $C_{22}H_{16}N_4O$. *Synonyms*.⁴

Benzeneazobenzeneazo- β -naphthol, $C_6H_5N=N-C_6H_4-N=N-C_{10}H_7OH$.
Tetrazobenzol- β -naphthol, Oil Red O, Cerasinrot, Scharlach R.
Fettponceau, Motirot 2R, etc. (Some are the methylated compounds.)

⁴ Merck's 1907 Index, New York, 3rd edition, 1907, 429.

It is a brick-red powder, insoluble in water, alkali, or dilute acid; slightly soluble in alcohol; very readily soluble in hot glacial acetic acid, chloroform, ether, paraffin, benzene, oils and fats, fatty acids, and all lipid solvents. It may be crystallized out of glacial acetic acid in sheeny metal green-brown plates, melting at 195°C.

When treated with strong sulfuric acid it gives a green color. It splits into amino-naphthol, aniline, and *p*-phenylenediamine when treated with tin and hydrochloric acid. The sodium salt of the sulfonated compound is known as B Scharlach, a much used water-soluble dye.

The peculiar solubilities of Sudan III have facilitated the study of fat digestion and absorption through the intestinal walls. It is even more soluble in bile than in fat, but the question of its solubility in soaps or glycerol does not seem clear. Pflüger⁶ states that the pure dye is freely soluble in bile and dissolves in both soap and glycerol giving a deep red solution. Mendel,⁷ while reaffirming its solubility in bile even in preference to oil, states on the contrary that it is insoluble in either glycerol or soap solution.

Bearing in mind the above properties we now turn to the examination of products on the market which have been in common use. The samples taken were those put out by (1) Kahlbaum, (2) Berlin Anilin Fabrik, (3) Grübler, (4) A. H. Thomas Co.

Specimen.	Melting point.	Color.	Remarks.
	°C.		
1	130	Brick-red powder.	Very readily soluble in oil.
2	120	Dark venous-red.	Solution in oil dark colored.
3	135	Dark brick-red, lumps showing lighter material inside.	Very readily soluble in oil.
4	120	Orange-red powder.	Solution in oil of deep orange color, more or less soluble in dilute alkalies and acids.
5	195	Green-brown sheeny plates.	Purified product.

⁶ Pflüger, E., *Arch. ges. Physiol.*, 1900, lxxxi, 375.

⁷ Mendel, L. B., *Am. J. Physiol.*, 1909, xxiv, 493.

These varying properties show unmistakably the presence of impurities in the commercial products.

Purification of Sudan III.

The dye may be separated from its impurities by first dissolving in alcohol or glacial acetic acid, and subsequently precipitating in water to remove all water-soluble substances; the repetition of this procedure results in a fine brick-red powder of low melting point similar to that of the commercial article. It should then be recrystallized out of hot glacial acetic acid three or more times, the purity of the fine sheeny flocculent crystals thus obtained being judged by the melting point. Very impure products need to be dissolved in a lipoid solvent and shaken out with dilute aqueous alkali and acid alternately until the aqueous solvent ceases to take up any further color.

The solubility of Sudan III in oil has been the subject of long discussion. Michaelis⁸ considered that it was readily soluble in oil, but does not state to what degree; on the other hand Mann⁹ considered the solution to depend on a chemical and not a physical process. Mann concludes from Michaelis' own work that the union between Sudan III and oleic acid is a chemical one depending on the oxidation of the unsaturated fatty compound, analogous to the action of osmium tetroxide, the only difference being that azo dyes form additive compounds with the fat without changing their color, while osmium compounds are readily decomposed.

The chemistry of Sudan III has been worked out in the elaborate researches of Michaelis. He showed that its staining power was dependent on the simple azo group "benzene azobetanaphthol," and varied according to the nature of the rest of the molecule.

Bell¹⁰ in his experiments on the interstitial granules of striated muscle secured his best results with Herxheimer's stain, and relative to the experiments here reported it should be noted that he states, "very variable results were obtained," indicating that the

⁸ Michaelis, L., *Virchows Arch. path. Anat.*, 1901, clxiv, 263.

⁹ Mann, G., *Physiological histology*, Oxford, 1902, 306.

¹⁰ Bell, E. T., *Internat. Monatschr. Anat. u. Physiol.*, 1911, xxviii, 297.

quality of the dye varied, although "only Grüber's dyes have been used." The methyl and ethyl compounds of benzeneazobenzene have a greater staining power—especially when no salt-forming groups are present. The "indifferent" character of Sudan III makes it a peculiarly efficient fat-soluble dye. It has great ability to unite with fat cells in both an acid and alkaline medium as compared with other dyes. This is shown by Robertson¹¹ in his work on tissue staining.

The solubility of Sudan III in oil has an important bearing on the relative availability of stained and unstained fat in the animal body. The assimilation of a solution of the dye would be expected to follow a different path from that of the assimilation of an oil compound, the latter possibly rendering the fat unavailable for use in the organism.

Unlike Scharlach R, Sudan III dissolved in alcohol and mixed with five to six volumes of water does not form a colloidal solution, and apparently does not show the same adsorption phenomena as do some other indifferent dyes. This property should be considered in the interpretation of the supposed urinary excretion of Sudan III.

Sudan III is easily detectable in the body fluids. Simple solution of the dye in alcohol gives a distinct pink color up to one part in ten million; likewise in any of the lipid solvents when it is present in traces (one part in a million). Gasoline, in preference to ether or toluene, was found to be the cheapest and best solvent. The writer did not find Salant's⁴ wool method satisfactory for dark colored urines. The wool adsorbs so much of the urinary pigment that it is almost impossible to tell whether there is any dye present. Sudan III is best extracted from lymph by a mixture of one volume of alcohol and two volumes of ether, a quantitative yield being obtained by shaking the mixture and standing it aside for 24 hours.

Absorption and Transport.

Mottram¹² showed clearly that animals possess varying degrees of ability to absorb and use fat; therefore one might expect

¹¹ Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.

¹² Mottram, V. H., *J. Physiol.*, 1915-16, 1, 380.

to find some variation in the results obtained by workers using different animals. Mendel worked chiefly with dog, cats, and rats; Salant used rabbits, the normal diet of which includes very little fat. In one of the following experiments the writer injected intraperitoneally an aqueous suspension of the dye into a rabbit and observed no excretion either in the urine or feces. The animal when killed showed a pink omentum, unstained below the surface, with scattered lumps of dye embedded in it. Intraperitoneal injection of the dye dissolved in oil gave good absorption and excretion, the dye appearing in the feces in 1 to 5 days. Rats injected intraperitoneally showed little absorption of the stained oil. The rats were killed after 2 or 3 weeks and there was found still present in the peritoneum a considerable amount of stained oil. Mendel³ has shown beyond question that when Sudan III is fed by mouth it passes readily into the lymphatics and thence into the blood stream; later it is eliminated with the bile into the alimentary tract.

There are other factors which influence the nature and rate of absorption of fat and therefore of stained fat. Various adjuncts have been used to assist in emulsifying the oil administered. Mendel used lecithin, a substance which Loevenhart and Souder¹³ showed to be an accelerator to the digestive action of pancreatic juice on oils. Salant⁴ used emulsions containing sodium carbonate, which would greatly decrease the solubility of the dye in the oil and so probably retard its absorption. Salant also used suspensions in gelatin, which might or might not be absorbed depending on the condition of the animal, such a preparation being comparable to a watery suspension.

In view of the above facts regarding the doubtful absolute purity of some of the preparations of Sudan III heretofore used, the question of the absorption of fat through the intestinal mucosa might well be brought forward again. As the dye has been relied upon as an exact index of the paths of absorption and transport of fat, any small amount of a foreign coloring matter present may give very misleading results, from which erroneous conclusions may have been drawn.

¹³ Loevenhart, A. S., and Souder, C. G., *J. Biol. Chem.*, 1906-07, ii, 415.

Excretion.

The work of Abel and Rowntree¹⁴ on the excretion of dyes leads one to expect Sudan III, on account of its indifferent character, to be excreted in the feces. This was found to be the case by Mendel³ and was confirmed in the present work on dogs, all of which were tested by the phenolsulfonephthalein test and found normal in respect to kidney function. Salant claimed that the dye was excreted in the urine, but obtained varying results from animals with deranged kidney mechanism, his experiments being conducted with rabbits to which very large doses of dye were given. The writer repeated this work with rabbits and found variation according to the purity of the dye, the size of the dose, and the kind of diet. In all cases *when the pure dye was fed, even in large amount, no dye was excreted in the urine*, soft deep red stools being obtained in 2 to 4 hours.

Small doses of the unpurified product given intraperitoneally did not appear in the urine. It was impossible to give to rabbits such large amounts intraperitoneally as by mouth. Large doses of the crude drug—as much as 2 gm. at one time—were administered with the aid of a stomach tube to full grown rabbits. During one or more days following, dark reddish brown urines were obtained. The dark red-brown pigment was not Sudan III. It was thought to be probably a foreign pigment present in the original impure dye, soluble in the body fluids, and excretable by the kidney in some form or other. This should not be interpreted as characteristic of the excretion of Sudan III. Pure Sudan III so administered to rabbits did not give pink urine; and the same negative outcome was observed when dogs were given moderate doses of the impure products with their daily food. The colored urines were only obtained when abnormally large doses of the crude dye with its impurities were administered to rabbits. The urines on standing deposited the pigment as a dark brown precipitate. The amount of the pigment seemed much increased when the rabbits were given exclusively an oat diet. Such a diet with its resulting acidosis changes the nature of the renal excre-

¹⁴ Abel, J. J., and Rowntree, L. G., *J. Pharm. and Exp. Therap.*, 1909-10, i, 231.

tion, and herein may lie the explanation of the appearance of the pigment impurity in the urine.

The *pure* dye is excreted in the feces only, the earliest appearance observed being after 2 hours. It is extractable by gasoline, whereby it may be estimated quantitatively.

Toxicity.

Weyl¹⁵ some years ago announced that diazo colors in general are non-poisonous. This has been confirmed in particular with regard to Sudan III by subsequent workers. Mendel stated Sudan III to be harmless even in large doses provided the dye was pure. Salant and Bengis in their experiments observed death after 1 to 3 weeks; but they regarded it as extremely doubtful whether death was due to the dye. Hatai reported that Sudan III retarded to a considerable extent the body growth of albino rats, and caused a striking change in weight of many of the organs, especially the liver, pancreas, thymus, testes, and ovaries.

The following experiments show that the *impure* dye may be markedly toxic. When 2 gm. were given at one time to normal rabbits, in all cases death ensued in a very short time. A neuritic-like condition of the hind legs developed first, and sooner or later the animals died in convulsions. Rabbits fed on an exclusive oat diet showed paralytic symptoms very rapidly after feeding the dye. The pure dye given in normal amount did not show any of these symptoms.

A number of albino rats were used to test further the toxicity of the various products. Those fed the crude preparations became very much debilitated. The orange colored dye (No. 4) stunted the growth, and brought about a general appearance of malnutrition; and in other rats the hind legs were paralyzed on one or both sides.

Path of Absorption and Excretion of Sudan III in Animals with Normal Kidney Mechanism.

Dogs were fed moderate doses of Sudan III in fatty food, and after several hours cannulas were inserted in the thoracic and bile

¹⁵ Weyl, T., *Die Teerfarben*, Berlin, 1889.

ducts to collect the lymph and the bile. Blood was taken from the femoral artery. Just before death the bladder was emptied with great care to avoid contamination, and the urine examined for the presence of the dye. All the animals were tested several hours before operation with the phenolsulfonephthalein kidney function test and were found to be normal.

The following results show that the dye administered with fat is well absorbed into the lymphatics, is transported into the blood, and excreted with the bile, and reabsorbed in it from the intestine. It is not excreted by the kidney, but always appears in the feces. Small doses of the crude products did not influence the color of the urine.

Experiment 1.—Dog, female, weight 13.5 kilos. Fed 4 gm. of Sudan III Specimen 1 during 1 week, at the same time adding plenty of lard to the diet. Very soft pink feces were excreted. On the 8th day, 3 hours after taking a heavy fat meal containing Sudan III the animal was operated upon to obtain samples of lymph and bile. Both contained a considerable amount of Sudan III. The milky lymph was intensely pink, and the dark bile, dried down with lime and extracted with ether, showed an abundance of Sudan III. The urine showed no trace of red color. At the conclusion of the operation 1 cc. of phenolsulfonephthalein was injected subcutaneously, and after 9 minutes 2 cc. of urine were obtained giving a strong positive reaction.

Experiment 2.—Dog, male, weight 14.3 kilos. 1 gm. of Sudan III Specimen 1 was added with 15 gm. of lard to the morning meal of ground biscuit. After 5 hours the animal showed signs of gastric disturbance; there was vomiting with diarrhea and it was several days before the animal appeared normal again. When fully recovered, the animal was given on 3 successive days 1 gm. of purified Sudan III with the morning meal. On the 3rd day, 1 hour after feeding, an operation was conducted to obtain lymph and bile. More than 100 cc. of a very pink lymph were obtained in 2 hours, and the bile collected was saturated with Sudan III. No dye appeared in the urine. The pure dye did not disturb the digestive system so much as the crude products.

Experiment 3.—Dog, female, weight 10.5 kilos. 0.5 gm. of pure dye was fed with 10 gm. of lard, and 2 hours later a similar amount was fed in half a pint of cream. After 4 hours the animal was operated upon as in Experiments 1 and 2. The lymph was decidedly pink, and the bile taken direct from the gall bladder when extracted with gasoline showed the presence of a considerable amount of Sudan III. There was no trace of dye in the urine. At the end of the operation 0.5 cc. of phenolsulfonephthalein solution was injected subcutaneously, and after 7 minutes it appeared in the urine showing that the kidneys were functioning well.

Experiment 4.—Dog, female, weight 12.4 kilos. Fed 1 gm. of pure dye, and after 2 days the dose was repeated with half a pint of cream. 1 hour after giving the second dose the dog was operated upon as before. The lymph flow was exceedingly small but quite pink in color. The bile gave a strong red color when shaken up with gasoline. Blood collected from the femoral artery was allowed to clot and the serum extracted with gasoline. It showed unmistakably the presence of the dye. There was no trace of dye in the urine.

Experiment 5.—Dog, male, weight 11.5 kilos. 1.5 gm. of Sudan III Specimen 1 with lard were added to the usual food. After 1½ hours an operation was conducted to obtain specimens of lymph, bile, blood, and urine. All except the urine showed the presence of Sudan III in marked amount.

Experiment 6.—Dog, female, weight 14.5 kilos. 1 gm. of Sudan III Specimen 4 was given with lard, cream, and biscuits. After 2 hours the body fluids were collected as in Experiment 5. The lymph and blood were strongly positive, but the bile was much lighter than usual containing only very faint traces of the dye. The urine, as in all of the experiments, showed no trace of Sudan III.

TABLE I.
Summary of Experiments on Dogs.

Experiment No.	Dye.	Sudan III in			
		Lymph.	Bile.	Blood.	Urine.
1	1	++++	++++	—	0
2	Pure.	++++	++++	—	0
3	"	++	++	—	0
4	"	+	++	+	0
5	1	++	++	++	0
6	4	++	+	++	0

The results with normal animals confirm the findings of Mendel and Daniels,³ and answer clearly the criticism of Salant and Bengis.⁴

To investigate further the nature of the absorption and excretion of Sudan III, experiments similar to those of Salant and Bengis were conducted on rabbits. The protocols of the various experiments bring out especially the toxicity of the crude products in common use.

Experiment 7. Toxicity of Commercial Sudan III, Specimen 1.—Rabbit 1, female, weight 1.6 kilos. Mixed diet (oats, corn, cabbage, and carrots). 84 mg. were injected intraperitoneally with 5 cc. of oil. During the following 3 days soft, deep red feces were obtained but the urine was nor-

mal. Similarly a week later 126 mg. were given in 10 cc. of oil. 8 days later 2.72 gm. in 20 cc. of oil were given by mouth. Intensely red soft feces were excreted in 3½ hours. Subsequent diarrhea made it difficult to keep the animal clean, the belly and hind quarters becoming smeared very red. The toxic symptoms of the dye developed gradually; after a few hours there was loss of control in the hind limbs and sprawling on the front legs, and the animal died in convulsions within 24 hours. No dye appeared in the urine at any time.

Experiment 8. Effect of Administering Sudan III, Specimen 1, Recrystallized Three Times, Melting Point 130°C., with Exclusive Oat Diet.—Rabbit 2, female, weight 1.76 kilos. The animal was fed on oats for several days, when the daily excretion of urine became very small. 92 mg. of dye were injected with 10 cc. of oil intraperitoneally. In a few days there developed paralysis in the left leg with albuminuria. The sickness continued acute as long as the exclusive oat diet was given. After 25 days carrots were added to the diet, and 2 gm. of the dye were given with 20 cc. of oil. A very heavy, dark brown urine was voided for 6 days, after which time the urine returned to its normal color, and the paralysis in the leg was much improved. The animal died several weeks later. The pigment in the urine was examined for bile pigment with a negative result. It precipitated out on standing. Hydrolyzed with acid and alkali it failed to give any red color. On account of its general properties and the fact that the administration of pure Sudan III did not produce this pigment, the pigment must have originated as an impurity in the commercial specimen of the dye.

Experiment 9. Toxicity of Commercial Sudan III, Specimen 3.—Rabbit 3, female, weight 1.7 kilos. Diet of oats and carrots. 2 gm. of the mixed impure residues from the recrystallization of the dye were given with oil by mouth. After 2 hours soft red feces were excreted, which in 4 hours changed to a red liquid stool. The urine collected during the following 6 days was a thick red colloid, after which time the animal appeared normal in every way. 12 days later 2 gm. of commercial Sudan III, Specimen 3, were given with oil by mouth. The animal excreted red-brown urine during several days, and gradually developed neuritic-like symptoms, the right side being first affected, and later the hind legs being completely paralyzed. At the end of the 3rd week the rabbit had a convulsion, and the following day died in convulsions.

Experiment 10. Effect of Administering Impure Sudan III, Specimen 4.—Rabbit 4, female, weight 1.84 kilos. Exclusive oat diet. 2 gm. of the dye suspended in water were given by mouth. On the following day only the feces were colored red, but a very dark brown urine was excreted for a week. After 3 weeks the animal was killed. It showed no stain either in the omentum or any of the fatty tissues of the body. The urine after standing a week deposited the pigment on the sides of the vessel. This brown material was examined as before, and was also apparently an impurity in the original dyestuff. There was degeneration of the liver. It was concluded that the dye failed to enter the circulation at all; otherwise at least the omentum would have been stained.

Experiment 11. Absorption, Distribution, and Excretion of Pure Sudan III Administered to an Animal on a Good Mixed Diet.—Rabbit 5, female, weight 2.0 kilos. Diet of oats, corn, cabbage, and carrots. 1 gm. was given by mouth with 50 cc. of oil. After 3½ hours very red feces were excreted and acute diarrhea followed, lasting several days. The urine was darker than normal, but the natural color returned in 5 days. The animal was killed a month later. The omentum, the fatty tissues of the neck and legs, and the kidney and heart fat all showed distinctly the presence of the dye, being stained pink. The nerves and spinal cord were quite white, and the bile was free from the dye. The pure dye was apparently non-toxic.

Experiment 12.—Repetition of Experiment 11. Rabbit 6, female, weight 2.1 kilos. Good mixed diet. 1.5 gm. of the purified material were given with 35 cc. of oil by mouth. There was absolutely no sign of any foreign pigment in the urine excreted during the following month. The dye was stored in the omentum and fatty tissues of the body, and excreted in the feces, as in the previous experiment.

Experiment 13. Effect of Injections of Small Doses of Pure Sudan III.—Rabbit 7, female, weight 1.72 kilos. Diet of oats, corn, and cabbage. 20 mg. of pure Sudan III were dissolved in 5 cc. of peanut oil and injected intraperitoneally. 5 days later 45 mg. in oil were injected subcutaneously, and after 13 days a water suspension of 65 mg. was injected intraperitoneally. There was no immediate appearance of the dye in either the urine or feces. The animal was killed a week later. It showed a heavily stained omentum, the inner layer of which was quite white. The particles of dye from the injection of the watery suspension appeared just scattered over the fatty surface, and were evidently unabsorbed.

Experiments were conducted further with white rats. The toxic effects of the crude impure dyes were most pronounced, being more marked from the preparation with a low melting point and abnormal properties.

Following intraperitoneal injection all specimens caused death in a relatively short time. In each case 200 mg. dissolved in 3 cc. of warm oil were used. If death did not take place the same day the animal was fed on a mixture of the stained oil and dog biscuit. In all cases the abdomen when opened gave off a foul smell. The omentum and intestine both inside and out were loosely coated with the dye. A control experiment conducted with the plain oil proved harmless.

A number of rats after fasting for 4 days were fed specimens of the commercial dye dissolved in oil and mixed with dog biscuit powder. There was produced a general debility and appearance of malnutrition. Specimen 4 was unlike the other crude speci-

TABLE II.
Summary of Rat Experiments with Sudan III.

Experiment No.	Dye specimen.	Dose.	Administration of dose.	Effect.	Remarks.
		gm.			
14	3	0.2 in 3 cc. oil.	Intraperitoneal.	Died in convulsions.	
15	3	0.2 in 3 cc. oil.	Intraperitoneal and in food.	Paralysis of hind limbs.	Died after 1 wk.
16	2	0.2 in 3 cc. oil. 0.5.	Intraperitoneal. In food.	Very debilitated.	Died after 12 days; empty stomach; stained oil not all absorbed from the peritoneum.
17	1	0.2 in 3 cc. oil.	Intraperitoneal.	" "	Died in 2 wks.; lost quick movement; hind limbs very slow.
18	5. pure.	0.2 in 3 cc. oil.	" "	Became bad tempered.	Continued to eat well; after 19 days gave Dye 4.
	4	<i>Ad libitum</i> .	In food.	Very debilitated.	Died 3 wks. later; loose hair; granulated ears; foul smelling, stained omentum and kidney fat.
19		Plain oil 3 cc.	Intraperitoneal.	No apparent effect.	After 1 wk. gave Dye 4.
	4	0.2 in 3 cc. oil.	"	Died in 3 days.	Stained oil mostly unabsorbed.
20	3	<i>Ad libitum</i> .	In food.	Debilitated.	Fasted 4 days, fed dye 3 days, then put on normal diet 1 mo., after which Dye 5 was given.

TABLE II—*Concluded.*

Experiment No.	Dye specimen.	Dose.	Administration of dose.	Effect.	Remarks.
20	5	gm. 0.2 in 3 cc. oil.	Intraperitoneal.	Died after 15 days.	
21	4	<i>Ad libitum.</i>	In food with oil for 1 wk.	Stunted growth, quite lively.	First fasted 4 days. During 8 wks. weight almost stationary, varying from 133 to 128 gm.
22	4	" "	" " "	" " "	Same as Experiment 21. varying from 168 to 153 gm. at time of death. Emaciated, very little body fat, no stained fat in the body.
23	3	" "	In food for 1 wk.	Debilitated, bad tempered till put back to normal diet.	Three rats grew normally up to nearly 300 gm. Killed after 8 wks. Omentum, neck, leg, and kidney fat heavily stained.

mens. The rats fed on it were very stunted in growth and did not show staining of the omentum and other fatty tissues of the body.

These experiments with the varied effects on the growth and health of the animal bring out the varied quality of the crude dye products, and confirm the results of the rabbit experiments which show the undoubted toxicity of the unpurified commercial preparations.

SUMMARY.

Sudan III dissolved in oil and administered intraperitoneally, subcutaneously, or by mouth, is absorbed, and may be traced in

the lymph, blood, and bile. It is transported to the fatty tissue of the body, being deposited particularly in the omentum. It is excreted in the feces, and is not found in the urine of normal animals.

Ordinary commercial preparations of Sudan III contain more or less impurity of a toxic nature. They may cause great debility, stunt the growth, produce a neuritic-like condition, and be fatal in large dose. The foreign substances may be excreted by the kidney, and appear in the urine as deep colored pigments.

The purification and properties of Sudan III are set forth, and their important bearing on problems associated with fat metabolism and vital staining discussed.

I am indebted to Professor Lafayette B. Mendel for assistance and suggestions in planning and carrying out the above experiments.

THE GLOBULINS OF THE JACK BEAN, *CANAVALIA* *ENSIFORMIS*.

PRELIMINARY PAPER.

By JAMES B. SUMNER.

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Cornell University, Ithaca.)

PLATE 1.

(Received for publication, November 13, 1918.)

While working with the urease of the jack bean, the author has had occasion to study the nature of the proteins that are present. The proteins of the jack bean have been investigated by Jones and Johns,¹ who claim to have isolated an albumin and two globulins. The precipitate obtained by half saturation with ammonium sulfate, present in small amount and having the greater sulfur content, they called concanavalin. The precipitate by complete saturation with ammonium sulfate, present in large amount and containing less sulfur, was named canavalin. The only reason Jones and Johns had for considering the jack bean to contain two globulins was the different sulfur content of these two fractions. Jones and Johns dialyzed jack bean extracts and found that spheroids were formed.

The author has found not two but three globulins present in the jack bean. Two of these globulins crystallize readily upon dialysis, while the third appears to be incapable of crystallizing. These three globulins can readily be separated by virtue of their markedly different solubilities.

The greater part of the precipitate by dialysis consists of spheroids and represents the uncrystallizable globulin; this is readily soluble in 1 per cent sodium chloride solution. It also dissolves in distilled water when the acid combined with it has been neutralized. The author believes that the name canavalin, applied by

¹ Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1916-17, xxviii, 87.

Jones and Johns to their precipitate by total saturation with ammonium sulfate, should be given to this protein.

The minute amount of material which crystallizes as needles is slowly soluble in 10 per cent salt solution and is not dissolved by alkali until an excess is present. The author has named this globulin concanavalin B.

The third globulin, crystallizing in bisphenoid form, and present in moderate amount, appears to be insoluble in any but concentrated salt solutions. The crystals when not recently formed probably have an outer layer of protean, and in this case will dissolve only in hot salt solution. If jack bean extracts are covered with toluene and simply allowed to stand exposed to the air for several weeks, this protein is precipitated as beautifully formed crystals having a diameter of about 0.1 mm. The author proposes to name this globulin concanavalin A.

When jack bean extracts are subjected to dialysis the bisphenoid crystals (concanavalin A) are precipitated first, canavalin separates next as spheroids, and the needles (concanavalin B) are formed last. A partial separation of the proteins can be made by filtering off the precipitates as they appear, but it is better to use the salt solutions named above. The author has never been able to accomplish any satisfactory separation by salting out with ammonium sulfate.

All three globulins give the usual protein color and precipitation tests when purified by several repetitions of dissolving and dialyzing; after such treatment the proteins give no Molisch test but may still contain phosphorus.

EXPERIMENTAL.

Extraction of Globulins.—The jack beans are ground as fine as possible in a coffee mill, and the meal is extracted over night with two and one-half times its weight of distilled water, toluene being mixed in as preservative. While salt solutions extract somewhat more of the proteins, the extra salt present considerably lengthens the time of the dialysis. The wet meal is wrapped in cheese-cloth and pressed in a tincture press. The milky juice is filtered by suction through a thick bed of filter paper pulp. A satisfactory filtrate can be obtained if the rim of the mat is firmly pressed down from time to time during the first part of the filtration.

Separation of Concanavalin A.—The filtrate is dialyzed against running distilled water, toluene being used as preservative in this as well as in all subsequent dialyses. The dialysis can be regarded as finished when a test portion of the material in the dialyser, on being filtered and added to distilled water containing a little dilute acetic acid, gives no haziness. The precipitate is filtered off by suction on an ordinary filter paper, weighed, and stirred to form a suspension with five times its wet weight of distilled water. Enough saturated sodium chloride solution is now added with stirring to give a salt concentration of about 10 per cent. The spheroid protein will dissolve at once, but the needles, the solubility of which depends upon size and age, may require as much as an hour for solution. Without waiting for this to take place, the material can be immediately started filtering by suction as the filtration will take a long time. The filter paper may clog up and have to be replaced by a new one.

The insoluble globulin remaining on the filter is washed with 10 per cent salt solution, transferred to a beaker, and dissolved with stirring in a very small amount of saturated sodium chloride solution at 40°C. Complete solution requires only a minute or so and can be observed by microscope. The denatured protein present is removed by filtration through filter paper pulp contained in a small Buchner funnel. If the filtrate is diluted with distilled water and allowed to stand, or else is dialyzed, bisphenoid crystals will be formed.

Crystals can be obtained by dissolving concanavalin A in the least possible amount of dilute ammonium hydroxide and neutralizing cautiously with dilute acetic acid; large crystals are formed if the original jack bean juice is poured into distilled water acidified with acetic acid, and allowed to stand a day or so. Large and perfect crystals can be formed by allowing solutions of the protein containing considerable ammonium sulfate to evaporate slowly, but the best method of preparing well formed crystals is to allow the original bean juice to stand, covered with a thick layer of toluene, in an open cylinder for about 1 month. Presumably the carbon dioxide of the air is slowly absorbed by the protein solution, with the formation of the insoluble globulin salt. Fig. 1 shows crystals prepared in this manner. Dr. A. C. Gill has been kind enough to examine them, and has provisionally

declared them to be "bisphenoidal in shape, probably belonging to the rhombic system, optically biaxial, with a large optical angle, and negative in optical character."

Separation of Concanavalin B and Canavalin.—The filtrate from concanavalin A, containing the dissolved spheroids and needles, is dialyzed, and the precipitated proteins are filtered off by suction and suspended in a considerable amount of distilled water. Concentrated sodium chloride solution is slowly added with stirring until all the spheroid globulin has dissolved. The needles are separated by centrifuging, washed with a little 1 per cent salt solution, centrifuged again, dissolved in 10 per cent salt solution, filtered by suction, and again dialyzed. Under favorable conditions many of the needles formed by dialysis are large enough to be seen easily with the unaided eye, having a length of about 2 mm. When the needles are treated with a few drops of 0.1 N ammonium hydroxide, they split up transversely, and the microscope will reveal hexagonal cross-sections. Fig. 2 shows two of these cross-sections together with some of the partly broken needles.

With excess of ammonium hydroxide the needles dissolve completely and are reprecipitated on adding acetic acid. Mineral acids in small concentration dissolve the needles instantly, but in higher concentration cause coagulation. The needles stain well with methyl violet after the addition of a trace of ammonia.

The uncrystallizable canavalin dissolved in dilute salt solution is precipitated by dialysis, and can be purified further by redissolving in dilute saline, filtering through filter paper pulp, and dialyzing once more. Instead of dissolving the canavalin in salt solution, one can suspend it in distilled water and add dilute ammonium hydroxide drop by drop until the neutral point is reached. All the canavalin will dissolve before the reaction becomes alkaline. This behavior of canavalin shows it to be a globulin of the legume type.

CONCLUSION.

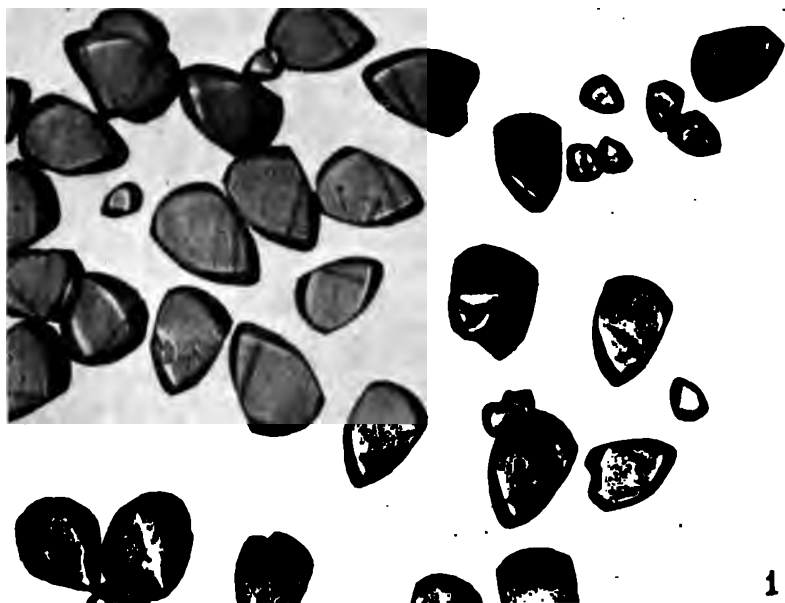
Two crystalline globulins and one non-crystalline globulin have been isolated from the jack bean. A method has been given for separating these globulins, and some of their properties have been described. More data will be published later.

The author wishes to express his thanks to Dr. B. F. Kingsbury for photographing the protein crystals, and to Dr. A. C. Gill for his kindness in pronouncing on the crystal form of concanavalin A.

EXPLANATION OF PLATE 1.

FIG. 1. Bisphenoids (concanavalin A). $\times 150$. Stained with alkaline methyl violet.

FIG. 2. Needles (concanavalin B). $\times 135$. Stained with alkaline methyl violet.



(Sumner: Globulins of jack bean.)

DO SEEDLINGS REDUCE NITRATES?

By J. DAVIDSON.

(From the Laboratory of Plant Chemistry, Bureau of Chemistry, Department of Agriculture, Washington.)

(Received for publication, November 11, 1918.)

In the course of studies on the nitrogen economy in plant growth, particularly with reference to wheat, it was desired to obtain some quantitative measurements as to the extent to which wheat seedlings reduce nitrates to nitrites. It was assumed on the basis of the experiments of Laurent¹ with peas, corn, lupine, and rye, which were repeated by Schreiner and Sullivan on wheat,² that seedlings possess certain principles in the nature of reductases which reduce nitrates to nitrites.

Experiment I.

Wheat seeds were germinated on a large floating aluminum disk. The seedlings at the age of about 1 or 2 days were transferred to smaller paraffined wire netting disks to which corks were fastened to keep them afloat in small agateware pans, each containing 500 cc. of sodium nitrate solution of a concentration of 1,000 parts per million. There were eight pans, four with 50 and four with 100 seedlings. The determination of nitrites in the solutions was begun the next day after the experiment was started and was made colorimetrically approximately every 24 hours. The results are given in Table I. As seen from this table, the quantity of nitrites fluctuated from day to day without any regularity. There was no tendency for nitrite accumulation, but there was an intermittent rise and fall in the nitrite content.

¹ Laurent, M. E., *Expériences sur la réduction des nitrates par les végétaux*, *Ann. Inst. Pasteur*, 1890, iv, 722.

² Schreiner, O., and Sullivan, M. X., *Studies in soil oxidation*, U. S. Dep't. Agric., Bureau of Soils, *Bull.* 73, 1910.

All this could be explained by the complexity of the biological processes going on at the same time: simultaneous reduction and oxidation, reduction beyond the nitrite stage, bacterial activity, etc. It was noted that there was no correlation between the number of seedlings and the quantity of nitrite produced, but that there was distinct correlation between the nitrites and the extent to which the seed portion of the seedlings came in contact with the solution in the pans, due to the tilting of the disks in the solution. The more the disks were tilted and the more as a consequence the seed itself came in contact with the solution, the larger was the quantity of nitrites found in the respective solutions.

TABLE I.

Reduction of Nitrates by Seedlings Grown in Nitrate Solution on Floating Paraffined Wire Netting Disks.

Pan No.	No. of seedlings.	Nitrites in parts per million.									
		Jan. 30	31	Feb. 1	3	5	6	7	8	9	10
1	50	0.75	0.5	0.33	5.0	5.0	5.6	5.0	5.0	8.0	6.5
2	50	0.75	2.0	2.2	6.0	6.0	8.1	8.7	11.2	19.0	15.0
3	50	0.87	2.4	3.1	4.0	5.0	5.6	9.4	11.0	15.0	2.2
4	50	0.75	2.0	2.2	4.0	5.0	4.5	2.5	4.0	9.0	3.1
5	100	0.88	1.4	Trace.	3.5	6.0	5.0	2.2	2.5	4.0	2.4
6	100	0.88	0.8	"	2.0	4.0	2.8	5.0	4.5	12.5	13.1
7	100	0.66	1.4	"	10.0	9.4	9.2	16.2	16.0	16.0	2.7
8		1.0	4.0	2.7	7.0	10.6	11.2	4.5	6.0	17.5	6.0

This observation led the writer to reopen the question as to the seat of the reducing principle, which according to Laurent¹ was equally present in the seed and in the attached plantlet.

Experiment II.

Wheat seeds were germinated on a floating aluminum disk as before. 50 seedlings were placed in each of two 400 cc. beakers. 100 plantlets were severed from the mother seeds. 50 of the severed seeds were placed in each of two similar beakers, and 50 of the detached plantlets were likewise placed in each of two similar beakers. 200 cc. of sodium nitrate solution of a concentration of 1,000 parts per million and 5 cc. of toluene were added to

each beaker. Toluene was selected as an antiseptic because in another experiment with ungerminated seeds it completely prevented the formation of nitrites in a nitrate solution. The reduction of nitrates by ungerminated seeds is assumed to be due to bacterial activity.

The beakers were covered with watch-glasses and were allowed to stand at room temperature for 24 hours. The respective solutions were then tested qualitatively for nitrites. Appreciable quantities of nitrites were found in the beakers containing the whole seedlings and also in those containing the detached seed. No nitrites were found in the beakers containing the detached plantlets.

This led to the conclusion that the seat of the reducing principle, at least in wheat seedlings, is exclusively in the mother seed attached to the young seedling.

Experiment III.

This experiment was set up to obtain quantitative measurements. Particular precautions were taken in producing the necessary seedlings. The aluminum disk and the pans were immersed in a solution of bichloride of mercury (1:1,000) for about 2 minutes. The seeds were soaked in a solution of bichloride of mercury (1:1,000) for 20 minutes. The seed, pans, and disks were washed with sterile water. The pan was filled with sterile water, and a sterile pan was inverted over the pan containing the floating disk and the seed.

When the seedlings were ready for use they were gently washed by decantation with water saturated with toluene. The experiment was then carried out in the same way as in Experiment II, differing only to the extent that Erlenmeyer flasks instead of beakers were used as containers. The Erlenmeyer flasks were stoppered with cotton plugs and sterile rubber stoppers.

No nitrites were found in any of the solutions containing the detached plantlets, the detached seeds, or the whole seedlings. No nitrites were found in any of these solutions after standing a week. A determination of nitrates showed the amount of nitrate in the solution practically unchanged.

The only possible conclusion from this experiment was that wheat seedlings do not possess any principles reducing nitrates to

nitrites. The failure to produce nitrites could not be ascribed to the toluene, for when no extraordinary precautions were taken in obtaining the seedlings, the whole seedlings and the detached mother seeds did produce nitrites with toluene. It could not be ascribed further to the previous treatment of the seed with bichloride of mercury, since the seed did not fail to germinate and since this treatment did not prevent the production of nitrites under the experimental conditions of Laurent.

The nitrites produced in Experiment II were evidently due to bacterial action. It would seem as though the same might also be said of the experiments of Laurent and especially of the experiment of Schreiner and Sullivan since they worked with wheat. It is a fair assumption that their precautions were not sufficient to guard against bacterial contamination.

The question arises why toluene prevented the production of nitrites in a nitrate solution with ungerminated seed, and why it did not prevent the formation of nitrites in Experiment II. A possible explanation is that the reducing organism works its way into the interior of the storage products of the seed, and it is thus protected against the action of the antiseptic. Or it is possible that some of the organic products originally present in the reserve material or formed during the process of germination are exuded into the solution and protect the reducing organisms against the antiseptic action of the toluene.

Experiment IV.

The question further arose why the toluene prevented the formation of nitrites in the solution with the detached plantlets in Experiment II. Was it because they did not furnish any food materials for the reducing organisms or because they did not offer the hypothetical protection against the antiseptic mentioned above?

To answer this question Experiment IV was carried out. Growing wheat seedlings were placed in the nitrate solution in such a way as to prevent any contact of the seed attached to the seedling with the experimental nitrate solution.

Tumblers of about 250 cc. capacity were used. They were covered with paraffined paper in which small holes were made

with a pointed glass rod. The rootlets of the wheat seedlings, obtained from unsterilized seed and without any precautions, were introduced through holes in the paper covers into the tumblers filled with the sodium nitrate solution, so that the seed and plumule rested on the paraffined paper without coming in contact with the solution at all. No precautions of any kind were taken to prevent bacterial contamination during the entire period of the experiment, which lasted about a week.

Only slight traces of nitrites were developed during the entire week. These traces may be safely disregarded. The reason that suggests itself is that the reducing organisms could not develop in the solution owing to the fact that the roots did not furnish enough foodstuffs for their growth. This experiment showed more clearly than any of the others that growing wheat seedlings do not reduce nitrates to nitrites.

Experiment V.

The next natural step was to test the nitrate-reducing ability of some other seedlings. Seedlings of barley, corn, oats, rye, and rice were obtained in the manner described above. Some were obtained from seeds previously sterilized with bichloride of mercury, and some without previous sterilization. The experiment was carried out essentially as in Experiment III. There were four Erlenmeyer flasks for each cereal; two contained seedlings obtained from sterilized seeds and two seedlings from non-sterile seeds. In each case, to one of the two Erlenmeyer flasks toluene was added as an antiseptic, the other being allowed to stand without any antiseptic. The tests were continued for about 10 days.

The addition of toluene completely prevented the formation of nitrites in every case when the seedlings were obtained from sterilized seed, except in the case of rice where a maximum of 2 parts per million of nitrites was observed. The previous sterilization of the seed was probably less effective in the case of the rice, due to its tight seed coats. The addition of toluene to the seedlings obtained from seed not previously sterilized, did not prevent the formation of nitrites but inhibited it considerably. The nitrites formed ranged from 1.2 to 3.2 parts per million. The

highest amount was again formed by the rice seedlings. The previous sterilization of the seed without the addition of toluene prevented the formation of nitrites for 24 hours only in the corn, but it retarded the formation of nitrites in every case. The nitrites for the first 24 hours ran from 5 to 20 parts per million, while in the seedlings, which were obtained without previous sterilization of the seed, the nitrites ranged from 8 to 70 parts per million.

These data would suggest the general conclusion that growing seedlings do not, as a part of their metabolic processes, reduce the nitrates in the outside medium in which they are growing.

The writer is indebted to Dr. J. A. LeClerc for valuable suggestions.

LOBULIN OF THE COCOANUT, COCOS NUCIFERA.

I. PREPARATION OF COCOANUT GLOBULIN. DISTRIBUTION OF THE BASIC NITROGEN IN COCOANUT GLOBULIN.

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The use of the cocoanut, *Cocos nucifera*, and cocoanut products has increased considerably in the United States in recent years. The fleshy endosperm is employed in a variety of ways by bakers and confectioners and also serves as a source of cocoanut oil. Before it was found necessary to divert tonnage for other uses, large quantities of copra were brought into this country for the preparation of cocoanut oil, and it is probable that its importation will be resumed as soon as the necessary tonnage is again available. Copra is prepared by removing the endosperm or cocoanut meats from the shells and then drying them in the sun or by means of artificial heat. It is then pressed to remove as much of the oil as possible. The residue or press cake, which contains about 20 per cent of protein ($N \times 6.25$) is ground and used as a cattle food.

The principal protein of the cocoanut endosperm is a globulin which has been studied by Ritthausen (1), Kirkwood and Gies (2), Chittenden (3), and by Osborne and his coworkers (4). Osborne and Harris (5) studied the distribution of the nitrogen in cocoanut globulin by Hausmann's method and obtained the following results:

	per cent
Amide nitrogen.....	1.36
Humin "	0.14
Basic "	6.06
Non-basic nitrogen.....	10.92

So far as we are aware, no previous attempts have been made to ascertain the distribution of the amino-acids in cocoanut glob-

ulin. We have prepared the globulin and found the distribution of the basic amino-acids by Van Slyke's (6) method to be as follows:

<i>Amino-Acid.</i>	<i>per cent</i>
Cystine.....	1.44
Arginine.....	15.92
Histidine.....	2.42
Lysine.....	5.80
Tryptophane.....	Present.

The percentage of free amino nitrogen in the unhydrolyzed protein was also obtained using the method of Van Slyke and Birchard (7). This was found to be 3.68 per cent, which is in fair agreement with one-half of the lysine nitrogen (3.21 per cent), as found in the hydrolysate of the protein. The value for cystine is of course low since some of the cystine is decomposed during the hydrolysis of the protein with hydrochloric acid. These results indicate that cocoanut globulin contains all the basic amino-acids necessary for maintenance and growth. This assumption is borne out by the results of nutrition experiments in which cocoanut globulin was the only source of protein in the diet for a prolonged period during which normal growth was obtained. The nutrition experiments will be published later. A complete hydrolysis of cocoanut globulin is also in progress.

In order to obtain the large quantities of cocoanut globulin needed for hydrolysis and for nutrition experiments, it was necessary to devise a method that would not be too laborious or time-consuming. This method is described below.

EXPERIMENTAL.

Preparation of Cocoanut Press Cake.—The fresh cocoanuts were punctured and the milk was drained out. The shells were then removed and the endosperm was cut into slices. These were dried in a vegetable drying machine through which circulated a current of air heated to 50°C. The oil was then removed as thoroughly as possible by means of an oil press of the expeller type. The resulting press cake was ground to a coarse meal, which contained 21.5 per cent of protein ($N \times 6.25$).

Preparation of Cocoanut Globulin.—About 2.5 kilos of the cocoanut meal were thoroughly mixed with 20 liters of a 10 per cent

sodium chloride solution and this mixture was allowed to stand for about a week at 1–3°C. in order to complete the extraction of the globulin. The mixture was then pressed in muslin sacks by means of a powerful press. The resulting turbid extract was again mixed with 2.5 kilos of meal and allowed to stand for a week at 1–3°C. The liquid was then pressed out as just described and filtered clear through paper pulp. The clear extract was dialyzed in parchment bags against running water for 7 to 10 days to remove chlorides. The globulin, which precipitated in a compact mass, was washed once by decantation with distilled water. It was then mixed with about 50 per cent alcohol and filtered off on a hardened filter, using suction. The globulin was then disintegrated and suspended in absolute alcohol. After standing for about 12 hours it was filtered off on a hardened filter and suspended in absolute ether. After standing in the ether over night it was filtered off as described above. The resulting globulin was placed in a vacuum desiccator over sulfuric acid to remove the ether. It was finally dried in a vacuum oven the temperature of which was gradually raised to 110°C. The protein was then ground to a fine powder in a mortar. The yield was 10 per cent.

Distribution of the Basic Nitrogen in Coconut Globulin by Van Slyke's Method.—Two 3 gm. samples of the protein were hydrolyzed for 28 hours by boiling each sample in 100 cc. of 20 per cent hydrochloric acid. Each sample was equivalent to 2.7957 gm. of moisture- and ash-free protein and contained 0.4860 gm. of nitrogen. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (8). The results of the analyses are recorded in Tables I and II.

Determination of the Free Amino Nitrogen in Coconut Globulin.—About 2 gm. of freshly prepared protein were redissolved in 50 cc. of 5 per cent sodium chloride. The free amino nitrogen was determined in duplicate in the usual manner using the Van Slyke micro-apparatus. Caprylic alcohol was used to prevent foaming and a correction was made for the gas evolved by reagents used. The results are recorded in Table III.

TABLE I.

Analysis of Cocoanut Globulin (Van Slyke Method).

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.....	0.0385	0.0391	7.92	8.05	7.99
Humin N adsorbed by lime.....	0.0063	0.0074	1.30	1.53	1.41
Humin N in amyl alcohol extract.....	0.0006	0.0004	0.12	0.09	0.11
Cystine N.....	0.0044	0.0049	0.91	1.01	0.96
Arginine N.....	0.1436	0.1432	29.55	29.46	29.50
Histidine N.....	0.0169	0.0189	3.48	3.89	3.68
Lysine N.....	0.0333	0.0291	6.84	5.99	6.41
Amino N of filtrate.....	0.2243	0.2174	46.15	44.73	45.44
Non-amino N of filtrate.....	0.0155	0.0292	3.19	6.01	4.60
Total N regained*.....	0.4834	0.4896	99.46	100.76	100.10

* Total nitrogen corrected for solubility of bases.

TABLE II.

Basic Amino-Acids in Cocoanut Globulin.

	I	II	Average.
	per cent	per cent	per cent
Cystine.....	1.35	1.53	1.44
Arginine.....	15.94	15.90	15.92
Histidine.....	2.28	2.55	2.42
Lysine.....	6.19	5.41	5.80

TABLE III.

Free Amino Nitrogen of Cocoanut Globulin Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	cc.	mm.	°C.	mg.	per cent	per cent
12.01	0.76	759	24	0.424	3.53	3.21
12.01	0.82	757	23	0.458	3.83	

SUMMARY.

1. A detailed method for the preparation of cocoanut globulin is described.
2. The basic amino-acids of the cocoanut globulin were determined by the Van Slyke method of analysis.
3. The free amino nitrogen was determined and found to equal nearly one-half the lysine nitrogen as determined in the Van Slyke analysis.

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SUPPLEMENTARY RELATIONSHIPS BETWEEN THE PROTEINS OF CERTAIN SEEDS.

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It is well established that the proteins from different sources differ greatly in their values for the support of growth. Gelatin fails to maintain nitrogen equilibrium when it serves as the sole source of protein in the diet of an animal, and since this protein fails to give certain of the amino-acid color reactions which are recognized as tests for the presence of protein, the faulty character of gelatin from the dietary standpoint was correctly attributed to the lack of tryptophane, tyrosine, and cystine. The development of analytical methods for the approximate determination of several of the amino-acids which result from the hydrolysis of proteins led to the accumulation of evidence that there are great differences in the yields of the several cleavage products into which the food proteins are resolved in digestion, and accordingly the conclusion was accepted that there probably existed marked differences in the nutritive values of the proteins of our several natural foodstuffs. This was demonstrated to be true for a number of isolated proteins by Osborne and Mendel (1). They conducted experiments which showed that the isolated proteins from many different sources vary greatly in their nutritive value. Their diets were not so planned as to make the results a measure of the relative values of the proteins studied.

In each of the natural foodstuffs of both animal and vegetable origin, there always occurs a complex mixture of proteins which in most cases differ markedly from each other both in solubility and in the proportions of the different amino-acids which can

be obtained from them on hydrolysis. It seemed probable that such mixtures of proteins should supplement each other so as to make the differences in the values of the different kinds of food with respect to protein less marked than in the isolated proteins themselves, and experimental trial has supported this view. The protein mixture of any one of the seeds, tubers, and other vegetable foods, which have been studied by satisfactory methods, have been found complete biologically, in that they furnish when digested all the cleavage products which are required for the construction of new body tissues. The differences in the values of the different natural foods with respect to protein depend therefore on quantitative variations. We have studied the protein values of all the more important seeds (2), and the potato as a representative of the tubers (3), and can compare these with milk, which has proved to be one of the best sources of proteins of high quality (4). It is not possible by chemical methods to obtain data of sufficient accuracy for comparing the values of the proteins. This is true because there are several amino-acids which cannot be determined beyond simple qualitative detection.

The biological method for the analysis of a foodstuff which was described by McCollum and Davis in 1915 (5) made it possible to determine with considerable accuracy, not only the comparative values of the proteins in individual foodstuffs, but also the value of a food with respect to each of its dietary factors. The comparison of the proteins from different sources warrants the following conclusions.

1. The best proteins for the support of growth in the rat are those of milk and of eggs. Meats likewise apparently furnish proteins of relatively high value (6), but these have not been sufficiently investigated. Recent experiments by Drummond (7) indicate that the coagulable protein of fish muscle has a relatively high value for the support of growth.

2. The proteins of the cereal grains have approximately one-third to one-half the value of the proteins of milk for the support of growth or maintenance in the rat. Experiments with growing pigs indicate that as much as 63 per cent of the protein of milk can be retained for the construction of new body tissue whereas the proteins of the cereal grains can be retained only to the extent of 20 to 26 per cent (8).

3. The proteins of the pea (9) and the navy bean (10) have apparently about one-half the dietary value for the support of growth in the rat that has been observed for the proteins of the cereal grains. We cannot as yet state whether the exact value indicated for the proteins of the navy bean should be accepted as final. It is possible that a cause contributing to the animals' failure to develop was the extensive fermentation of hemicelluloses which are contained in the bean, and which beyond question caused injury to the alimentary tract through constant and extensive dilatation. This factor is not responsible for a false conclusion regarding the poor quality of the navy bean proteins, for we have fed beans at lower planes of intake and have in these experiments, in great measure, eliminated fermentation as a serious factor. These results confirm the view that the proteins are actually of low value, but possibly are slightly better than the growth curves indicate. The proteins of the soy bean are distinctly better than those of the navy bean or the pea, but soy bean proteins appear to be no better for the support of growth than are those of the cereal grains. The soy bean contains about three times as much protein as do the cereal grains and can be fed at high planes of intake without evidence of the presence of anything injurious, and therefore appears from the results of certain feeding experiments to be of excellent quality (11), but when fed in amounts which furnish such planes of protein intake as can be secured with cereal grains, and properly supplemented in all respects, the proteins of this bean appear to be of no greater biological value than are those of wheat, oats, or corn.

4. Certain proteins are deficient in one or more of the essential amino-acids and are, therefore, incapable, when fed as the sole source of protein, of inducing any growth whatever in a young animal. These proteins do, however, possess remarkable values as supplementary sources of amino-acids for certain vegetable proteins, and thereby greatly enhance their values for the support of growth. Thus McCollum and coworkers (12) found gelatin or zein to be of great value when fed with the proteins of the oat kernel. Neither are of appreciable value when fed as supplements to the proteins of the corn kernel. Gelatin is an excellent supplement to the proteins of the wheat kernel, but zein does not enhance the value of wheat proteins to an extent

which could be demonstrated by their experiments. Zein, however, supplements the proteins of the pea in a very satisfactory manner (9).

Osborne and his coworkers have described the results of the application of the Fischer ester method for the determination of the individual amino-acids to numerous proteins of both animal and vegetable origin (13). These values, while not in most instances actually quantitative in nature, show clearly that there are wide variations in the yields of the digestion products, the amino-acids in proteins from different sources. From such studies the deduction is warranted that when two or more natural foodstuffs, each of which contains proteins not possessing high values for the support of growth, are fed together, it is probable that they will in some degree supplement each other's deficiencies, because of one furnishing somewhat more of that amino-acid which in the other forms the limiting factor for the support of growth.

In former papers we have thrown some light on the supplementary relationship between the proteins of certain seeds (14) and between gelatin and the proteins of certain seeds (12). The present paper is descriptive of further experiments in the same direction. Chemical methods when applied to proteins have contributed greatly to the advancement of theory, but the data regarding the supplementary relationships among the proteins from different sources must be obtained by actual observations on growth or maintenance of animals. The biological method for comparing the relative values of the proteins from certain sources has yielded results very different from the results of chemical investigations of the same proteins. The proteins of the pea and navy bean appear from the quantitative chemical data available to be better constituted than are those of the important cereal grains, but feeding experiments on growing animals show the legume seeds to be inferior to these. In the present studies we have sought to find the relative values of mixtures of proteins derived from two seeds, one furnishing one-third and the other two-thirds of the total protein of the food mixture.

Our studies of the dietary properties of the more important seeds have shown that when each of these is supplemented with

purified food additions so as to make it satisfactory for the support of growth at the optimum rate, except with respect to the protein factor which is not supplemented, a content of protein equivalent to 9 per cent of the dry matter in the diet suffices to maintain growth at rates varying from less to more than half the rate at which the young rat is capable of growing. In such experiments as we are considering the seed is supplemented with respect to both certain salts and fat-soluble A, since it has been demonstrated that aside from the quality of the proteins as a limiting factor in the seeds these two are the only factors which require modification in order to complete the seed from the dietary standpoint.

In our studies relating to the special properties of our natural foodstuffs, we recognize the limitations of our experimental methods. When an animal in infancy is fed a diet unsatisfactory in any respect, it does the best it can under the circumstances. There is a variable factor in the vitality with which the individual is born, which in some measure will determine its ability to utilize food faulty in any respect. Only in this way can we account for the great variation in the ability of several individuals in the same experimental group to grow on the same diet. Since it is not possible to determine the extent to which any individual possesses natural vigor, an experiment should be conducted with several animals so that one or more will be of great vitality. The elimination of the unfit in the breeding stock is important for this reason.

When the faults of a diet are of a certain degree of magnitude, animals with a fair amount of vigor may grow at the normal rate and reach full adult size and yet not be in a state of optimum physiological well being. If continued on such diets, they may be tardy in maturing sexually, or may be capable of but a fraction of the fertility of the vigorous, well nourished individual. It has likewise become apparent that animals which have grown at a satisfactory rate, appear vigorous, and produce a few young at or near the usual age, may fail to maintain fertility to the age normal for the species. In all cases where the diet is below the optimum in character the signs of involution characteristic of old age appear earlier than in the well nourished. Since these several variations from the normal in the usual functions

of the adult animal serve as valuable criteria as to the state of nutrition and are more refined than the simple observation of the growth curve, we have extended our observations in recent years to include them.

Another test of great value in determining the adequacy of a diet is to observe the ability of the mother to nourish successfully a litter of young reduced to a standard number (15). The nutritive undertaking of the mother rat with about four young is sufficient to cause failure in weaning them successfully within 30 days if there is anything radically wrong with her diet. With minor degrees of deficiency from any cause the growth of the young will be retarded or suspended. In the present studies we have made observations on all these criteria, in order to estimate as accurately as possible the relative values of the proteins of the different rations in those cases where a comparison of the growth curves of the experimental animals would show no differentiation in the state of nutrition for animals of different groups.

The rates of growth secured on 9 per cent of protein from each of several seeds, properly supplemented with respect to inorganic salts and fat-soluble A, are shown in Chart 1. This chart shows that much better growth was secured with a ration similarly constituted but containing 8 per cent of protein from milk powder than with 9 per cent of protein from wheat, rye, maize, flaxseed, barley, oat, or kafir, and that in a mixture of rye and flaxseed, in which rye furnished protein equal to 6 per cent and flaxseed furnished half as much, protein was secured which has superior biological value to an equivalent amount of protein from any single variety of seed yet examined. Indeed it appears that rye and flaxseed proteins in this proportion are nearly if not quite equal in value for growth to the proteins of milk. This conclusion is tentative and must be confirmed by further reproduction records, as well as by observations on the rate at which animals fed comparable amounts of protein from the several sources here compared develop the characteristics of old age.

The value which such studies have both in human nutrition and animal production can be readily understood. We now fully understand the nature of the supplements which must be added to a mixture of seeds in order to render it complete from the dietary standpoint, and also that when such mixtures are supplemented

with respect to all other factors, the rate of growth will be determined solely by the extent to which the proteins of the food can be transformed into body proteins. It is therefore, of fundamental importance that we should know just what foodstuffs when combined give mixtures whose proteins make good each other's deficiencies, and to what extent, in order that we may make the best use of our foodstuffs. The character of the curves shown in the charts makes evident the extent to which economic loss may result from feeding wrong combinations of foodstuffs in animal production.

We have shown by numerous experiments, both with growing young and with adult animals, that a liberal supply of all dietary factors over maintenance requirements supports well being better than does a parsimonious supply (16). It is desirable to have definite information as to whether optimum nutrition can be secured equally as well with certain relatively low planes of protein intake, where the protein is of excellent quality, as with proteins of lower value fed in higher planes according to their relative worth for transformation into tissue proteins. To determine this we shall present data later on the comparative values of mixtures of proteins derived from multiple sources. It is not possible to answer the question as to how much protein we need for the most satisfactory promotion of human nutrition until we know the quantitative supplementary values of all the combinations of the important types of foodstuffs, and have determined by experiments upon animals the plane of protein intake of known relative value as compared with proteins from other sources, which most effectively defers the signs of old age.

Our object in feeding protein at the plane of intake of 9 per cent of the dry food mixture in these experiments should be explained. As previously stated, such cereal grains as maize, rye (9), and barley (9, 17) contain proteins of such values that when fed at 9 per cent of the food mixture, supplemented with respect to certain salts and fat-soluble A, young rats are able to grow at approximately half the normal rate. We prepared the present series of rations each containing 9 per cent of protein derived from two seeds, one furnishing two-thirds and the other one-third of the total protein, and supplemented them in the necessary ways. The results show whether the protein mixture

in any case has lower or higher values than maize, rye, or barley, and to what extent. This plan, as will be seen from a study of the growth curves in the charts, affords an excellent method for the quantitative comparison of a series of proteins with respect to their biological values. 2 per cent of butter fat we have found to be sufficient for the maintenance of good growth when all other dietary factors are of good quality.

In the series of experiments reported in this paper we have employed the following combinations of proteins:

<i>Seed Furnishing 6 Per Cent of Protein.</i>	<i>Seed Furnishing 3 Per Cent of Protein.</i>
Barley.	Flaxseed oil meal.
Wheat.	" " "
Maize.	" " "
Pea.	" " "
Kafir.	" " "
Soy bean.	" " "
Rolled oats.	" " "
Cottonseed flour.	" " "
Millet seed.	" " "
Rye.	" " "
Soy bean.	Millet seed.
Wheat.	" "
Rye.	" "
Maize.	" "
Rolled oats.	" "
Pea.	" "

The most interesting point brought out by the growth curves in these experiments is the failure of the proteins of two seeds to supplement each other to any greater degree than they do in most cases. According to the present universally accepted theory of protein metabolism, the explanation of this failure must be due to the relatively low yield of each of the seeds in a mixture in some one or more of the essential amino-acids. Until our experiments with the combinations of seeds are completed we cannot draw conclusions as to which amino-acids are the limiting factors in these experiments.

Chart 1.—These curves illustrate the rate of growth secured with young rats on diets containing 9 per cent of their dry substance as protein, derived in each case from a whole seed. The

dietary factors other than protein were so constituted as to be fairly satisfactory for the promotion of good nutrition. (The composition of these rations was seed = to 9 per cent protein; NaCl, 1.0; CaCO₃, 1.5; butter fat, 3.0; dextrin to 100). For comparison the growth curves of young rats on a diet containing 8 per cent of protein from milk and another its 9 per cent of protein from a mixture of rye and flaxseed are presented. Both the latter sources of protein are of distinctly better quality than are any of the protein mixtures derived from a single seed. It was determined in the case of each ration that the limiting factor was the protein supply, and that on the addition of purified protein and nothing else much better growth and nutrition could be secured.

With a ration containing 9 per cent of barley protein the growth curve was not so good as with a similar ration containing wheat protein. There were two females in the experimental group, and although both were kept until they were 8 months old, and showed distinct signs of age, no young were ever reared by them.

The growth curves for the animals fed 9 per cent rye proteins were somewhat better than were those for the barley group, but not quite so good as in the wheat group. Two females produced three litters of young (eighteen young). Two litters were allowed to die soon after birth. The other, consisting of eight young, fared somewhat better. At 10 days there were seven left (weight, 71 gm.). At 32 days but two were alive (weight, 55 gm.). Both of these survived to the 39th day. The mothers looked very old at the age of 8 months.

With the diet containing 9 per cent of maize proteins, one female was isolated in a pregnant condition, but no young were ever seen. She doubtless ate them as soon as they were born (2).

With wheat proteins at 9 per cent, only an occasional litter of one or two young has been secured (2). None of these was weaned.

The animals in these experimental groups only rarely showed soreness of the ears and tails. In nearly all cases they were nervous and irritable. Their coats were always poor, but there was little loss of hair, except in the case of the young which lived after the development of a coat of hair. All showed early appearances of old age.

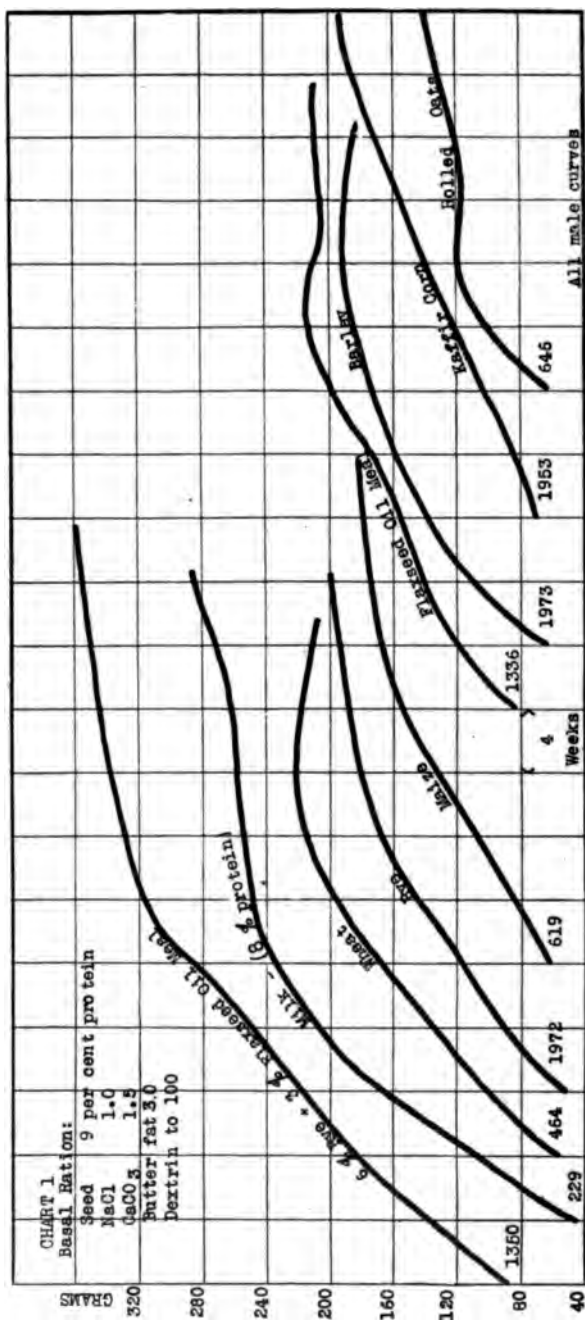


CHART 1.

In the experiments in which the curves for Lots 464, 619, and 646 were obtained, we employed complex salt mixtures instead of a simple mixture of sodium chloride and calcium carbonate, which we later found to be the only inorganic additions necessary for any of the seeds studied. These complex salt mixtures supplied, in the amounts in which they were employed, somewhat less calcium, sodium, and chlorine than we have later employed in supplementing these seeds. It is possible that with a more liberal supply of the three elements better nutrition might be secured with diets containing 9 per cent of wheat or maize proteins, but not sufficient to change in any marked degree the value of the comparisons made in this paper.

Each of these curves is representative of a group of four or five animals which were fed at the same time and in the same cage with the same ration. The curves show that a careful search for fortunate combinations of natural foodstuffs which will mutually enhance the values of each other's proteins, will yield results of importance both in human nutrition and animal production.

Chart 2.—Lots 1356 and 1350 illustrate the growth records of young rats fed a ration, the protein of which was derived from flaxseed oil meal (3 per cent) and rye (6 per cent). In the former case the mixture of seed products was supplemented with inorganic salts alone, and in the latter with both inorganic salts and butter fat to supply the fat-soluble A. The improvement resulting from the addition of butter fat is striking. The ration was, of course, of poor quality because of the character and amount of its proteins, and the improvement in well being which can result from supplementing a single dietary factor in a ration poor in some other respect emphasizes the principle which we have repeatedly enunciated; *via.*, the importance of considering the relative values of the several factors which operate to make the diet what it is. In order to secure the best possible growth curves with any plane of protein intake which is just above the actual requirements of the animal, it is essential that the remaining factors in the diet be close to the optimum in quality. In many human diets derived from wholesome natural foods, two or three dietary factors are more or less faulty in character. Under these conditions there will be a lowering of the ability to

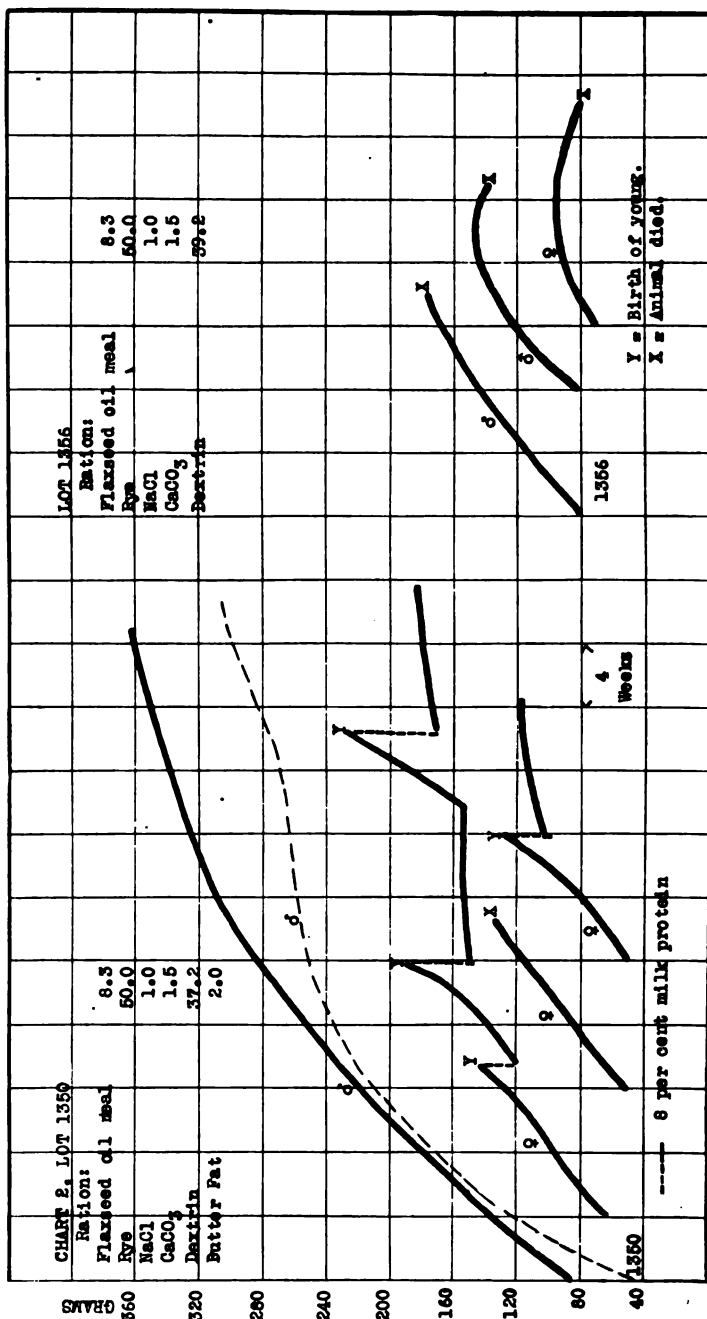


CHART 2.

perform the normal functions of life, such as growth, reproduction, and the suckling of young as well as would be the case if all dietary factors were of better quality.

Both the growth and reproduction records of Lot 1350 were better than for any others in the series to which we have fed 9 per cent of protein derived from two seeds. No other group has been so successful in rearing the young.

Three females produced six litters (thirty-four young) of which ten young were successfully weaned. They appeared normal in vigor, but were small for their age. The mothers maintained their weight while nursing. One of the second generation died while giving birth to young. All were in good condition at end of the experiment.

Chart 3.—Lot 1339 derived 6 per cent protein from rolled oats and 3 per cent from flaxseed oil meal. Their growth was approximately that which might be expected had they received 9 per cent of protein derived entirely from one of the cereal grains, and corresponded to about half the rate at which the young rat is capable of growing.

The single litter of young born (seven young) were finally weaned but were undersized. At 56 days of age the seven together weighed but 313 gm. These young grew but little when confined to the diet of the mother. This further supports the view that there is a progressive loss of vitality under such a dietary régime as these animals were subjected to.

For comparison with the preceding lot, the curves of Lot 1336 are presented. These rats were fed 9 per cent of protein derived entirely from flaxseed oil meal. Two females produced each a litter of young. One mother destroyed her litter of six young soon after they were born. The other litter of four young weighed 107 gm. at the age of 34 days. They should have reached this weight when about 22 days old. The curves of two of this litter are shown in the chart. The young were not able to grow appreciably on this diet.

Lot 1344 received a diet containing 9 per cent of protein, 6 per cent of which was derived from millet seed and 3 per cent from flaxseed oil meal. Growth was very slow on this diet although it was fairly satisfactory with respect to all dietary factors other than protein. For comparison the curve of growth for a male

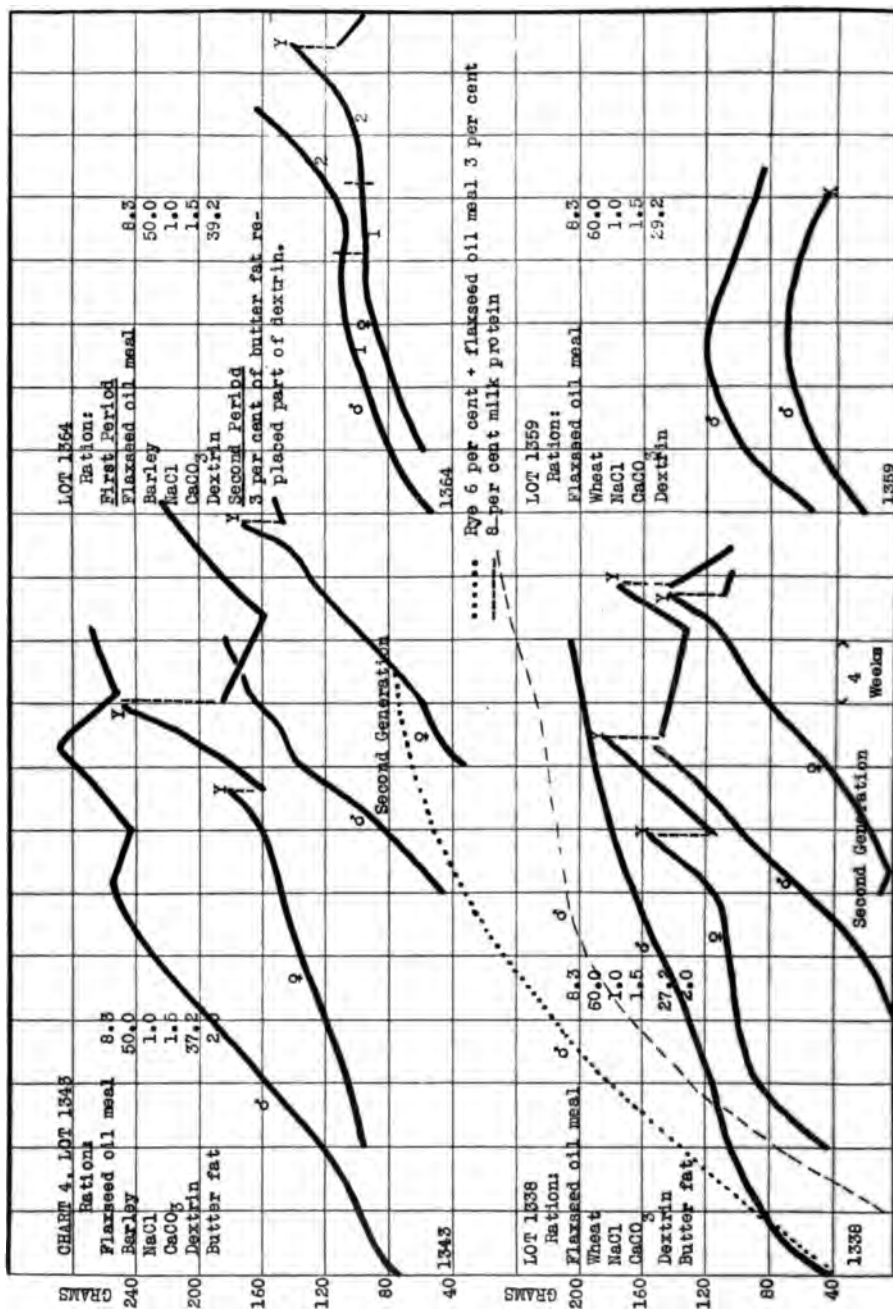
rat fed 8 per cent of protein derived from skim milk powder, and that secured with a diet containing 9 per cent of protein derived from rye and flaxseed oil meal, are placed in contrast with those of Lot 1344, and take the place of normal curves.

Lot 1365 was fed a diet containing 9 per cent protein and from the same source as in Lot 1344. In the case of Lot 1365 the grains were supplemented only with respect to inorganic salts, whereas in the diet of Lot 1344 both salts and butter fat (fat-soluble A) were added. The rate of growth was somewhat accelerated by the fat-soluble A. That the limiting factor in this diet was the character and amount of the protein is shown by the rapid growth in Period 2, when a part of the dextrin was substituted by 12 per cent of purified casein. Millet seed, cottonseed, or oat proteins are not supplemented by the proteins of the flaxseed to anything like the extent that rye proteins are.

Chart 4.—Lots 1343 and 1364 illustrate respectively the effects of feeding such a mixture of barley and flaxseed oil meal as would furnish a diet containing 9 per cent of protein, two-thirds of which came from barley and the one-third from flaxseed. In the one case it was supplemented with both inorganic salts and fat-soluble A (in butter fat), and in the other with inorganic salts only. The value of the protein mixture derived from barley and flaxseed in these proportions is not sufficiently high to enable the animals to grow on a diet containing 9 per cent of protein, at a rate faster than they could do on a similar diet in which the protein is derived solely from one of the cereal grains, as wheat or maize kernel. However, the reproduction records are in nearly all cases better on the mixed proteins than on a seed alone.

Two females in Lot 1343 gave birth to three litters of young (twenty-seven), and of these nine were weaned. No young were ever weaned on wheat or maize alone on diets of this character. Their growth was approximately half normal. Five young at 50 days of age weighed 260 gm. The mothers rapidly developed the characteristics of senility while nursing their litters.

In the second period of Lot 1364, there was a tardy response with renewed growth after the introduction of butter fat into the diet. Recovery is not always to be expected when the faults of a diet are corrected after suspension, because of lowered vitality in the animals.



Lots 1338 and 1359 illustrate the great difference in the growth curves of two groups of rats which were fed diets containing but 9 per cent of protein, two-thirds of which came from wheat and the remainder from flaxseed oil meal, due to the addition of butter fat, as compared with what could be secured without it. With such rations it is possible to improve the nutrition of the animals either through the addition of protein or of fat-soluble A. The growth curves of young rats on 8 per cent of protein from milk and on 9 per cent protein from a mixture of rye and flaxseed (Lot 1350, Chart 2) are inserted as normal curves. Two females in Lot 1338 produced three litters (eighteen) young. Of these twelve were weaned. The nursing mothers lost weight during lactation. One dropped from 195 to 178 gm. in 44 days, during which time the young grew to a collective weight of 227 gm. The young were always small for their age and the mothers early showed signs of old age. That there is a fair supplementary value here is especially shown in the reproduction records. (Compare growth of second generation with Chart 3, Lot 1339 and Lot 1344.)

Chart 5.—Lot 1337 received a diet in which the content of 9 per cent of protein was derived from maize (6 per cent) and flaxseed oil meal (3 per cent). The diet was satisfactorily constituted with respect to all other dietary factors. Growth was slow, and the animals failed to reach the full adult size.

Two females were included in the experimental group. One of these never had any young, while the other had two small litters (six young), only two young of which were weaned, and these were small for their age. These animals were extremely timid and nervous.

Lot 1347, in Period 1, were fed a diet which was fairly satisfactory in all respects, except for the quality of its protein. The diet contained 9 per cent of protein, 6 per cent from peas (cooked and dried), and 3 per cent from flaxseed oil meal. In Period 2, 2 per cent of calcium phosphate was added to see whether the addition of more phosphorus would enhance the value of the diet. The results showed that this was not the limiting factor, but that the failure of the animals to grow at a better rate was the result of the insufficiency of the protein supply. The one litter of young produced were eaten by the mother. The de-

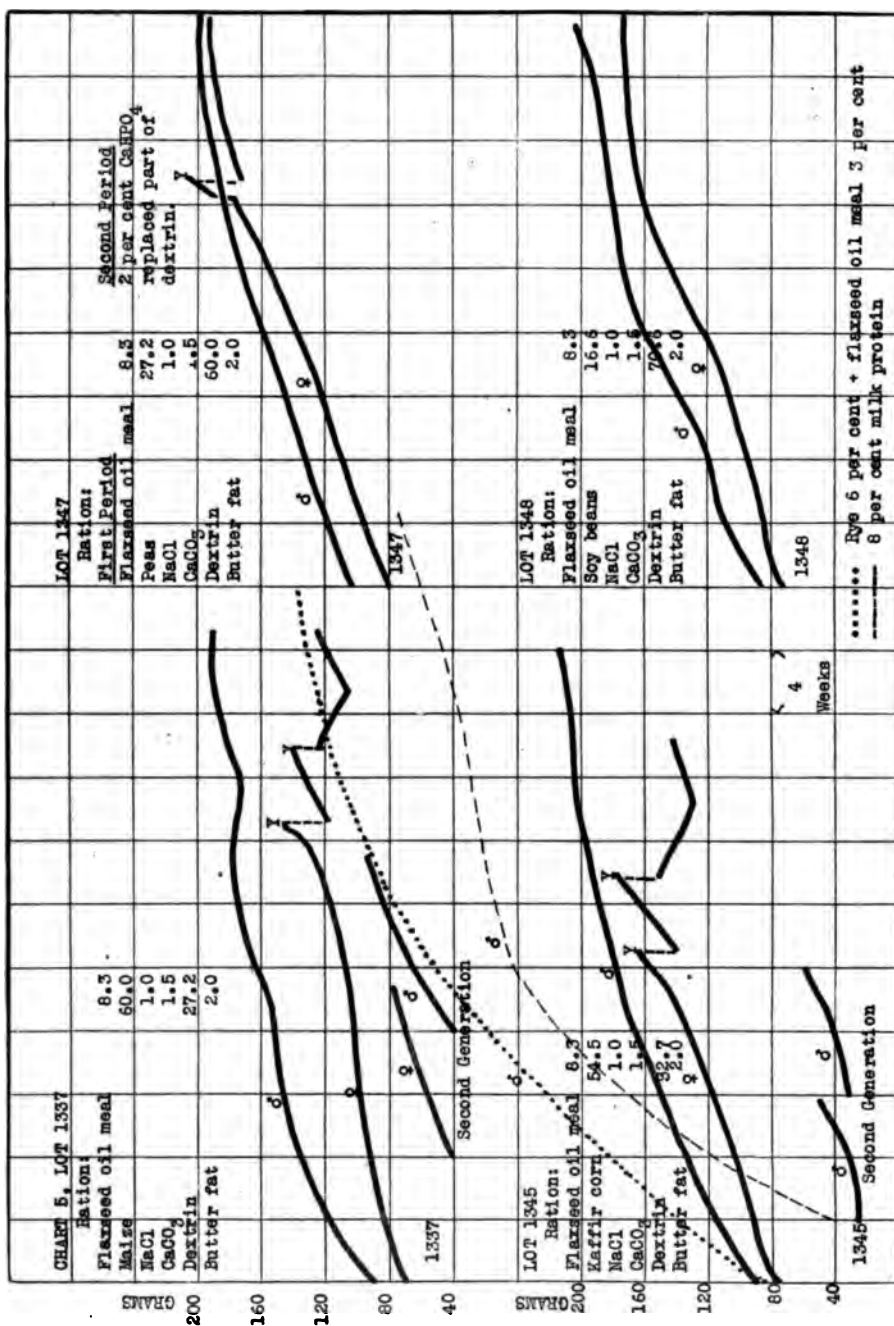


CHART 5.

struction of the young by the mother rat is almost, without exception, the result of malnutrition.

Lot 1345 which was fed a diet containing 6 per cent of protein derived from kafir and 3 per cent from flaxseed oil meal failed to grow at any better rate than is possible on a similar diet in which the same plane of protein intake is furnished from one of the cereal grains. There is some evidence that the combination of the proteins from these two sources makes an improvement in their biological value. One female never produced any young, but the other had two litters (ten young). Five of these were successfully weaned, while the remaining litter was destroyed by the mother. All were very much stunted. At the age of 53 days they weighed collectively 127 gm. After weaning, the young never grew on the mother's diet.

Lot 1348 grew slowly on a diet in which the 9 per cent of protein was derived from soy bean (6 per cent) and flaxseed oil meal (3 per cent). No young were produced by this group. It is evident from the growth curves that this combination of proteins is of relatively low biological value for growth.

Chart 6.—Lot 1378 which was fed a diet containing 9 per cent of protein, two-thirds of which came from peas and one-third from millet seed, proved to be much better nourished than they would have been with the same amount of protein derived solely from one of the cereal grains. This is brought out especially by the ability of the second generation to grow as well as did the first, and to produce and rear young. Two females of the first generation, and a daughter of one of these produced six litters (twenty-five young) of which fifteen were reared. Although none of the females reached a non-pregnant weight greater than 175 gm., they were always in good condition. One female aged rapidly after the birth of her third litter of young. It is evident that we have in this combination of proteins from two seeds a supplementary relation of some importance.

Lot 1376, which derived its 9 per cent of protein from rolled oats (6 per cent) and millet seed (3 per cent), shows that again we have a supplementary relation between the proteins of one seed for those of the other, which distinctly enhances their value, but the improvement is less than in the case of a mixture of peas and millet seed (Lot 1378). Three females produced three litters

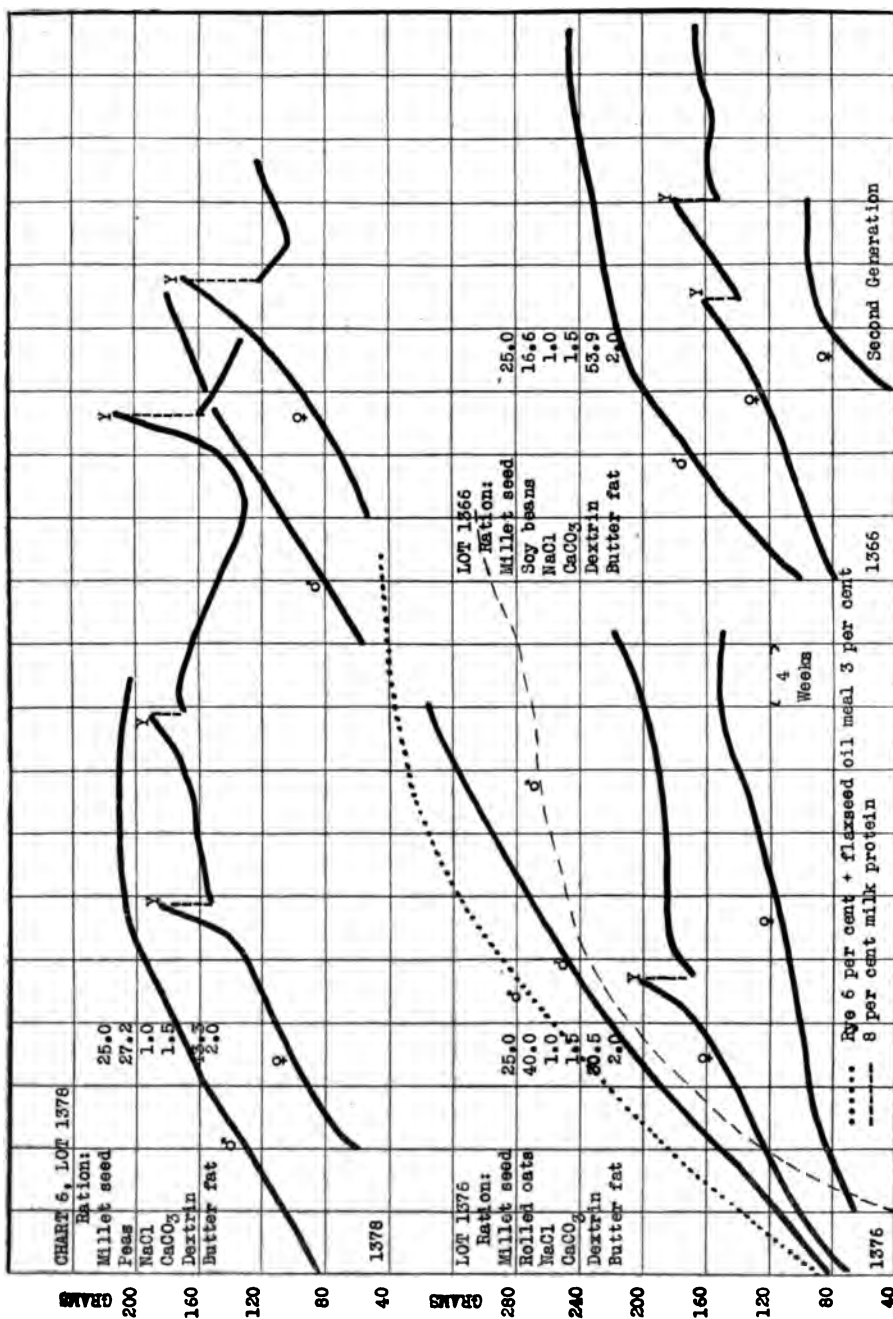


CHART 6.

of young, all of which were destroyed by the mothers within the first 3 days after birth. The ability of different individuals to grow on this diet varied considerably, which is in harmony with our observations on faulty diets in general.

From the character of the curves of Lot 1366 it is evident that a mixture of the proteins of millet seed and soy beans when combined is little if any better than the proteins of wheat alone, or any of the other cereal grains alone. They are better, however, than we have been able to secure with 9 per cent of soy bean protein alone. Great variation in the capacity of different rats to grow on this diet was observed. There were three females in the group, and from these six litters were secured (thirty young). Ten of these were reared but were always undersized. The mothers all maintained their weights while nursing their young, which serves as evidence that this protein mixture was better than the proteins of one of the cereal grains taken as the sole source of protein.

Chart 7.—Lot 1374, which received 9 per cent of protein, two-thirds derived from wheat and one-third from millet seed, grew about as they would have on the proteins of one of the cereal grains alone at the same plane. The difference in the value of the proteins of this mixture as compared with those of the cereal grains alone is seen in the reproduction records. Whereas on wheat proteins at this plane of intake we have secured almost no young, an occasional litter of one to three being born, in the present case two female rats produced four litters (twenty-six young), and succeeded in rearing eighteen young. All remained undersized. We feel certain that such a reproduction record could not be secured with *this plane* of intake of proteins from any single cereal grain, satisfactorily supplemented in every respect except with regard to protein. The mothers in this group all maintained their body weights while nursing their young.

Lot 1375 derived its protein, which constituted 9 per cent of the diet, from rye (6 per cent) and millet seed (3 per cent), the mixture of seeds being properly supplemented with respect to other factors. The growth curves represent a little better than half the rate at which the young rat is capable of growing. Two females produced four litters of young (fourteen young) of which

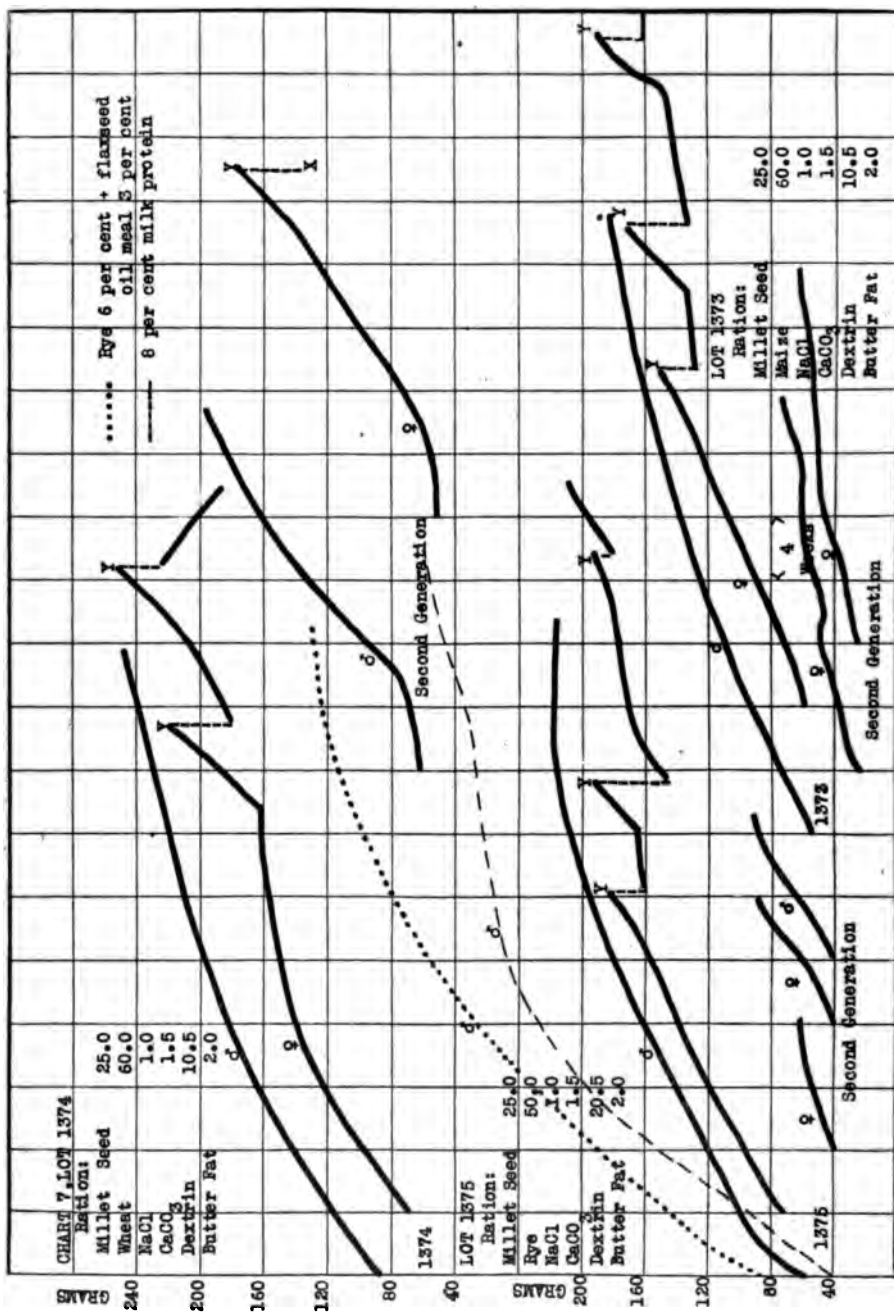


CHART 7.

only three young were successfully reared, and these were undersized. The mothers lost weight while nursing their young and aged very rapidly. This evidence, together with the character of the growth curves, indicates that there is little enhancement of the proteins of one of these seeds by those of the other. The young, after weaning, were able to grow to some extent on the diet of the mother.

Lot 1373 was fed 9 per cent of protein from a mixture of maize (6 per cent) and millet seed (3 per cent). The growth curves indicate very little tendency, if any, of the proteins of one of these seeds to supplement those of the other. This is also supported by the reproduction records. Three females produced five litters (twenty-seven young) of which but three young were weaned and these were dwarfed by undernutrition. The mothers did not maintain their body weights while nursing their young. It appears, therefore, that there is little improvement in this mixture over the proteins of the individual seeds as the sole source of protein. All animals in this group aged early. Their young were almost hairless and very timid. None ever grew appreciably when confined to the mother's diet.

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STUDY OF THE METABOLISM IN MULTIPLE EXOSTOSES.

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That multiple exostoses are hereditary in character appears to be established by the observations of Jungmann (1), Gibney (2), Percy (3), and many others. It had not been clear, however, until recently that these tumors were congenital in character (Vaughan, 4), as they are not usually noticed until they attain an appreciable size. With the aid of the x-ray these growths have in many cases been shown to be present shortly after birth in children with exostotic parents. They are usually not noticed before the child is from 4 to 6 or more years of age, but they seldom, if ever, appear after the skeleton is fully developed. Males appear to be much more susceptible to the disorder than females (Fischer, 5). A female free of exostoses but belonging to an exostotic family may yield offspring which are affected. Ehrenfried (6) gives the ratio of males to females affected as 3:1.

There are a few cases on record where multiple exostoses were associated with rickets (Grünfeld, 7; Stolzenberg, 8), but this is not usually the case, and according to Vaughan there has been no demonstration of a direct connection between the two diseases. In fact it has been pointed out that the arrangement of the cartilage cells in growing exostoses is different from their arrangement in rickets. Close intermarriage has been observed by Meyer (9) in a case where no heredity of the disease could be proved, the father of the patient being the uncle of the mother. Ritter (10) noticed in one case that the thyroid gland was small and barely palpable. As this gland has an important influence on bone growth or at least on the metabolism of calcium, a closer observation of the condition of the thyroid in this disease may be

of interest. Lortat and Sebaréanu (11) and others have looked for a connection between tuberculosis and multiple exostoses. The tuberculosis theory, however, lacks any definite substantiation. As has been pointed out, tuberculosis is so common that there are sure to be some patients who have tuberculosis in addition to multiple exostoses. Recent authors consider the cause to be a disturbance in the bone-forming cartilage, though the real etiological factor is unknown. For discussions of the clinical aspects of the disease the papers of Vaughan and Ehrenfried may be consulted.

No attempts have been made so far as we are aware to determine by metabolic studies whether multiple exostoses are primarily a disorder of metabolism or whether the disorder shows from this standpoint any analogy to rickets. As a contribution to this phase of the subject, the present work was carried out on a patient in the Jefferson Hospital in the service of Dr. Hobart A. Hare, to whom we are indebted for the clinical history and for the opportunity of making this investigation.

Clinical History.—B. G. Female, age 15 years; native of Russia. Admitted to Jefferson Hospital, May 6, 1915, and discharged without change of condition June 26. Father, mother, eleven brothers and sisters all living and well. No history of cardiac, renal, pulmonary, chronic joint disease, or cancer in family. Patient never had any serious illness. Menses began at 14 years of age; about 2 months apart; no pain associated. Chief complaint related to the bony growths on the bones of the arms and enlargements of the finger joints. When the patient was 5 years old she began to notice an enlargement on the left radius which gradually increased. At 12 years of age there developed an enlargement on the middle finger of the right hand—middle phalanx. Some nodules appeared at about the same time on the ankles. At this time one of the nodules was removed from the radius.

The growth of these nodules was very slow and without pain. The only thing that the patient complained of was the deformity which went with the bony nodules. The patient was apparently suffering from a gastrointestinal disturbance also, as she passed 77 stools from May 15 to June 15, the number per day varying from one to eight. During the 7 days of the experimental period twenty-five stools were passed. This did not appear, however, to be a matter of discomfort or complaint.

Physical Examination.—Fairly well developed female. Eyes, ears, nose, mouth, and neck normal. Thorax fairly well developed. Abdomen normal, no tenderness and no masses felt. Extremities: There were a number of bony growths on the lower ends of the radius and ulna, with some thickening of bone generally. There was also a thickening of the digits with a tendency to dislocation due to atrophy of ligaments. There were some bony growths on the inner side of the ankles, similar to those on the radius and ulna. Weight: May 17, 84 pounds; May 24, 82 pounds; June 4, 81 pounds; June 11, 81 pounds; June 23, 81 pounds.

Method of Study.

The patient was kept in a ward of the hospital in charge of a special nurse and placed on a uniform diet which consisted of 500 cc. of milk, 90 gm. of soda crackers, 50 gm. of corn flakes, 100 gm. of meat, 30 gm. of potato chips, and 1,000 cc. of water per day. The milk was sampled every morning. The sample was poured into an evaporating dish, and evaporated at 100°C. Each succeeding day's sample was poured into the same dish, and at the end of the period the evaporation was continued to constant weight. The residue was ground up in a mortar and preserved in a glass stoppered bottle for analysis. Enough of the crackers, corn flakes, and potato chips were put aside for the whole period which necessitated but one sampling. The meat was also prepared for the whole period previous to the experiment, thoroughly mixed, boiled and again mixed, placed in glass jars, sterilized, and kept in a frozen condition until used. A carefully prepared sample was evaporated to constant weight and kept for analysis.

The urine was collected in exact 24 hour samples which were preserved by refrigeration and thymol. The feces were separated by the use of carmine. The stools as passed were collected in a friction top can which had previously been rinsed with a 10 per cent alcoholic solution of thymol and dried. The can containing the feces was kept in the refrigerator until the experimental period was over. As the sample was semiliquid in character because of water added in transferring, it was evaporated to dryness before analysis. The evaporation was carried out under

a pressure of 250 to 300 mm. and a temperature of 50–75°C. The gases coming off were first passed through standard acid and then through an alkaline lead solution to catch any hydrogen sulfide coming off. The total alkalinity of the gases calculated as ammonia nitrogen was 0.206 gm. or less than 0.4 per cent of the total nitrogen excreted during the experimental period. The lead sulfide formed was oxidized to lead sulfate and the sulfur estimated as barium sulfate. It amounted to 0.081 gm. of barium sulfate or 0.0113 gm. of sulfur, or less than 0.003 per cent of the total sulfur excreted. It is obvious, therefore, that the evaporation of feces did not incur the loss of an appreciable amount of these elements.

McCrudden's methods were followed for the determination of calcium and magnesium. Sulfur in the urine was determined by Benedict's method. Sulfur in the feces and the food was determined by a modification of the sodium peroxide fusion method which will be published later. Phosphorus was determined by Neumann's method, and nitrogen by the Kjeldahl method.

The experimental period lasted for 7 days, from May 29 to June 5.

DISCUSSION.

The most obvious abnormality brought out by the balance of the five elements (Table I) included in our study is the loss of 21.8 per cent of the nitrogen ingested. One might expect that this was entirely due to faulty digestion or absorption associated with the disturbed peristaltic action, but this cannot be the case as more than 87 per cent of the excretion of nitrogen was through the urine—a percentage not far from normal. It is obvious that the body is losing protein, which is borne out by the fact that the patient's body weight was slightly decreasing at this time, although the diet was higher in nitrogen than that of the free choice, preexperimental period. This loss of nitrogen and lack of appetite of the patient were no doubt associated with the gastrointestinal disturbance. It is somewhat surprising that the sulfur balance is positive, as sulfur excretion also commonly parallels protein metabolism. As the disease in question is characterized by an abnormal production of cartilage, which is known to be rich in sulfur, it is possible that the sulfur ordinarily set

free in the decomposition of body protein is retained for this purpose.

There is also a distinct retention of calcium, magnesium, and phosphorus. But the relative distribution in the excretion of these elements between the urine and feces is about normal. The calcium ratio for normal persons varies between 1:4.5 to 1:9, while we found 1:7.4. There is normally more magnesium found in the urine than in the feces, and we observed the same in this case; namely, 0.7988 gm. in the urine and 0.7552 gm. in the feces. From two-thirds to three-fourths of the phosphorus is found in the urine in normal persons, while we found 59.2 per cent in the urine, which is only slightly abnormal. It is interest-

TABLE I.
Metabolic Balance in Multiple Exostoses..

	CaO	MgO	P ₂ O ₅	S	N
Total amount ingested, gm.....	6.400	1.747	13.546	4.480	44.68
Excretion in urine, "	0.635	0.798	7.660	3.460	47.46
" " feces, "	4.695	0.755	5.286	0.661	6.99
Total excretion, "	5.330	1.554	12.946	4.121	54.45
Retention, gm.....	1.070	0.193	0.600	0.4354	—
Loss, "	—	—	—	—	9.76
Retention, per cent.....	16.7	11.0	4.4	9.55	—
Loss, " "	—	—	—	—	21.8
Average daily gain, gm.....	0.153	0.028	0.086	0.062	—
" " loss, "	—	—	—	—	1.39
Total excretion found in urine, per cent.	11.9	51.4	59.2	83.9	87.2

ing to compare the per cent retention of these elements with those found by McCrudden and Fales (12) in a growing boy. We found that calcium, magnesium, and phosphorus were retained to the extent of 16.7, 11.0, and 4.4 per cent respectively, while McCrudden and Fales reported 17, 22.4, and 25 per cent respectively. It is not known at what stage in the development of the body the intake and output of these elements balance each other. It is not likely, however, that a balance would be reached at the age of 15, and as the ratio of the urine-feces excretion is approximately normal, it is probable that the metabolism of these elements is about normal. That absorption from the intestine was not quite so complete as usual was to be expected from the rapid-

ity with which the food residues passed through the bowel. Our metabolism study also supports the view that there is no connection between rickets and multiple exostoses as the urinary output of the ingested calcium has been found seldom to reach 5 per cent, more often being less than 1 per cent in rickets, while we find in multiple exostoses a normal excretion of 10 per cent.

Bergeim, Stewart, and Hawk (13) found pronounced daily variations in the urinary excretion of calcium and magnesium in their metabolism study in acromegaly and they suggested that this might be due to an accumulation in the intestine of residual material rich in salts which are soluble with difficulty. As in our study the intestines were well swept out, it is interesting to note that there was much more regular output of these elements. The

TABLE II.
Daily Excretion in Urine.

Day of experiment.	CaO	MgO	P ₂ O ₅	S	N
	gm.	gm.	gm.	gm.	gm.
1	0.083	0.123	1.178	0.542	7.54
2	0.101	0.144	1.000	0.509	6.24
3	0.084	0.117	1.149	0.508	7.07
4	0.097	0.116	1.097	0.474	6.81
5	0.094	0.106	1.057	0.444	6.38
6	0.087	0.183	0.998	0.410	5.86
7	0.090	0.109	1.182	0.574	7.56

authors mentioned above (13) report an excretion of calcium oxide on 3 consecutive days of 0.190, 0.085, and 0.225 gm., and of magnesium of 0.250, 0.043, and 0.191, while our greatest variations for calcium for 3 consecutive days were 0.0828, 0.1012, and 0.0836 gm., and for magnesium 0.1229, 0.1441, and 0.1169 gm. (Table II).

A question naturally arises as to the existence of a relationship of the exostosis to the marked peristaltic activity of the intestine which might be expected to affect the general nutrition of the patient. This possibility may not be entirely ruled out by the failure of previous observers to note the presence of such disturbances in their cases, as, not being a matter of complaint in our patient, it might have been previously overlooked. It appears more likely, however, to be merely one of the numerous complications which have been found associated with multiple exostoses.

CONCLUSIONS.

A study was made of the metabolism in a case of multiple exostoses. A loss of nitrogen was noted and in part ascribed to the associated hypermotility of the intestine. A retention of sulfur was observed and attributed to storage in the growing cartilage. The retentions of phosphorus, calcium, and magnesium appeared to be within normal limits as did also the percentage excretions of these elements by way of the urine.

The findings do not support the view of a relationship of this disorder to rickets or to any primary disturbance of general metabolism.

The authors desire to thank Dr. P. B. Hawk for the opportunity of carrying out this work and for his suggestions.

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THE VITAMINES IN GREEN FOODS.*

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There is at present little available information which enables one to judge the relative amounts of vitamins furnished by the leaves of plants. Owing to the prevailing high cost of food products of this type the use of them may become greatly restricted, so that if they actually furnish an important part of the vitamin supply of our diet much harm may result from lack of them. Under present conditions, therefore, it is important to know the real nutritive value of the green foods in common use. If these serve no other purpose than to gratify the palate their use can be dispensed with without further concern, whereas if they furnish an essential addition to our dietary, care must be taken to supply a sufficient amount of them, and their cheapest sources should be ascertained.

In the case of the seeds of the more important cereals and of some of the legumes we already have information which affords an approximate estimate of the quantities needed to supply enough of the *water-soluble* vitamin for the normal growth of young albino rats. There are also similar data concerning numerous animal food products.¹ Some information respecting the

*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ For references to the literature see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309; 1918, xxxiv, 17. Drummond, J. C., *J. Physiol.*, 1918, lii, 95.

distribution of the *fat-soluble* vitamine in food products of animal or vegetable origin has been obtained; but apart from indications of its relative abundance in the fats of milk, eggs, liver, and kidney, our present knowledge is still scanty.¹

It has been stated that the green leaves of plants are especially rich in the fat-soluble vitamine² so essential for the growth of young animals, but the data on which this assertion is based are insufficient to justify quantitative conclusions of other than a very general character. For example, McCollum and his associates have shown that on a diet containing 10 per cent of immature alfalfa leaves as the source of all vitamins a young female rat grew slowly to 130 gm. during 5 months. Another young rat on a similar diet containing only 5 per cent of the alfalfa leaves lived for 2 months, but did not grow. When the quantity of alfalfa was increased to 30 per cent rapid growth and reproduction followed. Apart from statements by McCollum³ that cabbage and clover furnish enough water-soluble vitamine for normal growth when the ration contains 15 to 20 per cent of the dried leaves, we have been unable to discover any further information relating to the content of this vitamine in green leaves, although numerous assertions have been made that they are especially rich in it.

In analyzing McCollum's data it is to be noted that while 20 per cent of dried alfalfa leaves permitted normal growth, 10 per cent led to slower growth and 5 per cent to none at all. We might, therefore, conclude that quantities between 10 and 20 per cent were sufficient to furnish enough of each of the vitamins needed; however, we cannot decide from these experiments whether growth was limited by a deficiency of the vitamine soluble in water or of that of the fat-soluble type. Consequently no opinion can be formed as to the richness of this plant in either one or the other of these essential food factors.

In view of the practical importance of definite knowledge of this subject and the paucity of available data we have under-

¹ McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333. McCollum, E. V., *Harvey Lectures*, 1916-17, xii, 151; *J. Am. Med. Assn.*, 1917, lxxviii, 1379. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxx, 13.

taken to study this question. Owing to the urgent need for such information we have been led to publish this preliminary account of the results thus far obtained. More detailed reports including reference to numerous other vegetable products will follow.

When any natural food contains more than one type of essential accessory it is necessary to test for each one separately if the relative content is to be ascertained. Thus in order to learn the minimum amount required to furnish enough of the fat-soluble vitamine for growth, the product to be tested must be fed in combination with a diet rich in water-soluble vitamine; and *vice versa* the minimum quantity required to furnish an adequate supply of the water-soluble vitamine for growth can only be determined when the basal diet contains sufficient of the fat-soluble type.

As a preliminary to such an investigation it is important to know whether the methods employed in preparing the various substances to be tested have any deleterious effects upon their vitamins. The initial content of vitamins in fresh green leaves cannot be established owing to their high water content, which makes it practically impossible to feed definite quantities during the long periods involved in properly conducted experiments. Therefore it is difficult to obtain direct evidence of changes which may be caused by drying. Such evidence as we have, as well as that reported by others, indicates that the water-soluble vitamine is stable at temperatures below 100°, and perhaps even at considerably higher temperatures. Thus it has been shown³ that wheat embryo, milk whey, and crude lactose from which the vitamine has not been fully removed, can be heated for 1 hour at 15 pounds pressure without apparent deterioration of the water-soluble vitamine, and we can confirm the statement that protein-free milk can be boiled for several hours without destroying its activity as a source of this essential food accessory. McCollum, Simmonds, and Pitz⁴ have also found that navy beans still retain much, if not all, of their value as a source of water-soluble vitamine after being heated in a moist condition at 15 pounds pressure for 1½ hours. In addition we have shown that butter fat through which live steam was passed

³ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

⁴ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.

for 2½ hours was still efficient as a source of the fat-soluble vitamine,⁵ but that the butter "oil" lost its potency if it was allowed to stand at room temperature for several months.⁶ Recently Steenbock and his coworkers⁷ have maintained that very prolonged heating of butter fat tends to diminish and eventually to destroy its value as a source of fat-soluble vitamine. In their behavior towards heat, the water- and fat-soluble vitamins must not at present be confused with the so called antiscorbutic factor in foods, which seems to be quite easily destroyed by even moderate heating.

Preparation of Plant Products.

The freshly gathered green leaves were carefully freed from adhering impurities, cut into small pieces, and dried at about 50–60° in the air, and then finely ground. The preparation of spinach included both the leaves and stems; that of cabbage only the sound inner leaves, which were separated from the stalk; those of the young alfalfa, clover, and timothy grass included the entire plant as obtained by mowing. These latter were cut in May when the alfalfa was about 20 inches high, the clover about 10 inches, and the timothy about 24 inches. All these plants were green and growing vigorously when cut.

Spinach Leaves as a Source of Water-Soluble Vitamine.

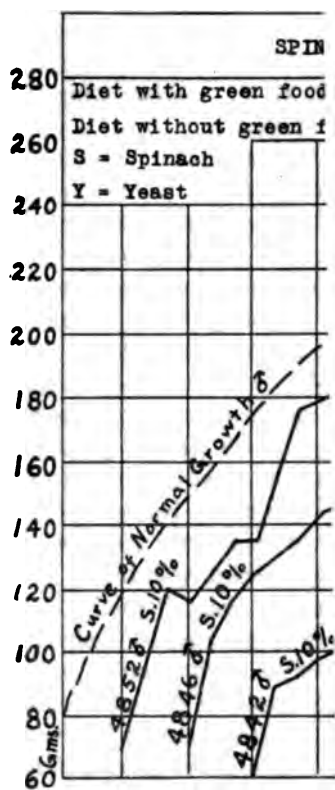
Chart I shows the results of feeding an otherwise adequate diet in which both the water-soluble and the fat-soluble vitamins were supplied solely by 10, 5, or 1 per cent of dried spinach leaves.

Those animals which received 10 per cent at first grew at a normal rate, but after 2 or 3 weeks grew more slowly. After being fed on this diet for 70 to 90 days the four animals responded promptly with a more rapid rate of growth when 200 mg. of yeast per day were furnished instead of spinach as a source of the water-soluble vitamine. From these results we conclude that 10 per cent of spinach supplies somewhat less than

⁵ Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 381.

⁶ Osborne and Mendel, *J. Biol. Chem.*, 1916, xxiv, 37.

⁷ Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.



Spinach...
 Meat resic
 Salt mixtu
 Starch....
 Lard.....

* The p
 xv, 317.

enough of the water-soluble vitamine needed for normal growth. Unless the food intake is known, experiments of this kind afford no information respecting the absolute amount of the vitamine-containing constituent of the food needed by the normally developing animal. The quantity actually consumed by the rats in these experiments can be estimated from Table I. From these figures it appears that from 4 to 7 gm. of dry spinach were eaten per week, or a total of 66, 71, 72, and 63 gm. respectively during the 13 weeks in which the rats received spinach. According to our experience about 1.4 gm. of dried brewers' yeast per week are required to promote normal growth, or 18 gm. during 13 weeks. Dried yeast is therefore somewhat more than four times as efficient as dried spinach leaves when used to supply water-soluble vitamine. We have already found that when used as a source of water-soluble vitamine the quantity of whole wheat, soy beans, dried eggs,⁸ or milk solids⁹ required to promote normal growth is approximately twice as large as is the quantity needed of the dried spinach.

When only 5 per cent of dried spinach furnished all the vitamine, practically normal growth was made during the first 2 weeks. Growth then became slower and ceased much sooner than when 10 per cent was fed. That the rats receiving 5 per cent of spinach were suffering from a deficiency of the water-soluble vitamine is shown by their prompt recovery when yeast was added to their ration. The failure of Rat 4841 to continue growth as long as Rats 4836 and 4857, on the same diet, is due to the fact that after the 1st week it ate very much less food than the other two and consequently received proportionately less of the spinach (see Table I). Its subsequent rapid recovery when supplied with more vitamine shows that on a suitable diet it was able to gain weight with great rapidity. When the food contained only 1 per cent of dried spinach growth soon ceased (Rats 4955 and 4956). Rat 4955 resumed growth for a short time when the quantity was increased to 5 per cent, and both these rats grew steadily after yeast was substituted for the spinach.

The marked difference in the curve of body weight for Rat 4841 compared with curves of the others on the 5 per cent spin-

⁸ Osborne and Mendel, unpublished data.

⁹ Osborne and Mendel, *J. Biol. Chem.*, 1918, xxxiv, 537.

TABLE I.
Weekly Food Intake, in Gm., of Rats on Diets Containing Spinach.

Rats....4852	4846	4842	4850	4857	4836	4841	4935	4856
Period 1 Spinach 10%	Period 1 Spinach 10%	Period 1 Spinach 10%	Period 1 Spinach 10%	Period 1 Spinach 5%	Period 1 Spinach 5%	Period 1 Spinach 5%	Period 1 Spinach 1%	Period 1 Spinach 1%
60	69	56	66	52	45	46	49	42
74	68?	57?	71	60	59	43	38	38
46	48	69?	54	49	51?	35	27	34
46	49	70	28	48	50	32		28
46	48	35	42	56	52	27	Period 2 Yeast	Period 2 Yeast
48	53	51	44	53	57		0.0 daily Spinach	0.2 daily*
55	47	70	41	52	46	Period 2 Yeast	5%	30
63	50	62	46	49	49	0.2 daily Spinach	33	Period 3 Yeast
48	56	42	55	46	36		36	0.0 daily†
43	44	39	52	44	Period 2 Yeast	5%	35	24
44	57	69	35?	Period 2 Yeast	0.1 daily Spinach	64	32	
46	59	62	34	0.1 daily Spinach	5%	52	Period 3 Yeast	
40	64	44	Period 2 Yeast	86	94?	Period 3 Yeast	0.2 daily	
			0.1 daily 72			0.0 daily		
Period 2 Yeast 0.2 daily	Period 2 Yeast 0.2 daily	Period 2 Yeast 0.2 daily						

Spinach 0% 107 108	Spinach 0% 84 88	Period 3 Yeast 0.2 daily Spinach 0% 92 95	Period 3 Yeast 0.2 daily Spinach 0% 94 82	Period 3 Yeast 0.0 daily Spinach 5% 66 65	41 52 Period 4 Yeast 0.2 daily Spinach 10% 91	Spinach 0% 47 60 Period 4 Yeast 0.4 daily 72 82 84 92	Period 4 Yeast 0.2 daily Spinach 0% Lost.
				Period 4 Yeast 0.2 daily Spinach 0% 72 84			
				Period 5 Yeast 0.4 daily Spinach 0% 84 96 98 95			

*During last 4 days.

† " " 2 "

each diet, illustrates occasional differences encountered in feeding individual animals on identical diets containing a fixed proportion of the vitamine-bearing ingredient. If for any reason the food intake of an animal falls below the normal, the amount of vitamine consumed likewise falls, with the effect of impairing the condition of the animal and further reducing its food intake. Thus it goes from bad to worse, for the influence of the vitamine is continuously diminishing and consequently also the food intake. This is by no means an isolated example, for our records contain numerous cases of this character. Table I, which gives the amount of food eaten each week during the various periods of these experiments, shows how great an increase in food intake results when the supply of vitamine is increased, the absolute amount eaten usually being doubled or trebled. The importance of an adequate supply of vitamine is especially exhibited by the four rats on the 10 per cent spinach diet. During Period 1 these rats were already receiving enough vitamine to promote growth at a nearly normal rate. The increased vitamine supply furnished in Period 2 very greatly increased the quantity of food eaten (see Table I).

Whether the water-soluble vitamine simply increases appetite and hence promotes growth, or whether it supplies some essential chemical factor which renders growth possible cannot be determined by any data heretofore recorded. The fact remains, however, that the addition of vitamine to a ration containing too little of this factor is followed by a very marked increase in food intake with accompanying rapid gain in weight. On the other hand, loss of weight on vitamine-poor diets almost invariably precedes a lessened intake of food, from which it seems more probable that the impaired health of the animal is due to insufficiency of an essential food factor rather than to an insufficient quantity of food. Conversely, when the water-soluble vitamine is increased in quantity after a decline on a vitamine-poor diet the increased food intake which follows is most likely due to an improved physical condition caused by the vitamine, for the resulting rapid recovery in body weight must necessarily be accompanied by a correspondingly greater food intake.

300
280
260
240
220
200
180
160
140
120
100
80
60
40

Fat-Soluble Vitamine in Spinach Leaves.

It is too early to make final statements as to the relative amount of the fat-soluble vitamine in spinach leaves, but the experiments here described indicate that these are rich in this food factor, so important for the normal growth of young rats. Even after 190 days of experimental feeding the four rats supplied with the 10 per cent spinach have shown no lack of the fat-soluble factor in their diet, as evidenced by a decline of weight or affected eyes, although after the first 80 to 90 days of feeding the spinach was removed from their food and nothing else supplied an appreciable amount of this type of vitamine. Rat 4852 at the present time has attained a weight nearly 50 per cent above normal while the other three are of practically normal weight. Rats 4836 and 4857, similarly receiving only 5 per cent of spinach, or 34 and 26 gm. during the first 87 and 83 days respectively of the 180 days of experimental feeding, are still normal in weight, although they received none of the fat-soluble vitamine during the last 93 and 97 days respectively.

These facts indicate that spinach leaves are in reality richer in this food factor than most of the products used in our ordinary rations. The final outcome of these experiments, together with others now in progress, must show how small a quantity will actually supply all that the young rat needs for normal growth.

Water-Soluble Vitamine in Cabbage Leaves.

Chart II shows that when 15 per cent of dried cabbage supplied both the water-soluble and the fat-soluble vitamins growth ensued at slightly less than the normal rate. It is possible that the failure of Rats 4616 and 4639 to make better growth was due to a deficiency in the fat-soluble factor, for when 9 per cent of butter fat replaced a corresponding quantity of lard an appreciable change in the rate of growth occurred. However, this failure was more likely due to a deficiency of water-soluble vitamine, because Rats 4642 and 4643, also receiving 15 per cent of cabbage, continued to gain in weight for a much longer time and recovered, after they had ultimately failed, when a little yeast was added to their diet. This recovery occurred in spite of the fact that no butter fat was fed during the 180 days of the experi-

TABLE II.
Weekly Food Intake, in Gm., of Rats on Diets Containing Cabbage.

Rate.....4616	4639	4642	4643	4658	4839	4855
Period 1 Cabbage 15%	Period 1 Cabbage 15%	Period 1 Cabbage 15%	Period 1 Cabbage 15%	Period 1 Cabbage 5%	Period 1 Cabbage 5%	Period 1 Cabbage 5%
41	38	51	49	43	61	37
48	52	54	48	44?	52	32
40	52	51	52	44	42	26
44?	54	58	57?	45	29	30
47	42	62	57	44		
61	42	69	64	47	Period 2 Cabbage 5%	Period 2 Cabbage 5%
53	42	67	60	55	29	35
51	37	62	60	47	27	31
51	36	64	52	47		
65	37	51	42		Period 3 Yeast 0.2 daily	Period 3 Yeast 0.2 daily
57	45	62	50	Period 2 Cabbage 10%	Period 3 Cabbage 5%	Period 3 Cabbage 5%
69?	45	72	58	67?	61	69
73?	54	75	68	64		
		73	67	70	Period 4 Cabbage	Period 4 Cabbage
		68	69?			
		76	52			
		70	66			
		57	63			
		44	60			
			73			
			83			
			72			
			58			
Period 2 Cabbage 15%	Period 2 Cabbage 15%	Period 2		Period 3 Yeast 0.2 daily		
61	41					
60	57					
82?	61					
77	79					
87	86					

95	95	Yeast 0.1 daily Cabbage 15%	Period 2 Yeast 0.2 daily Cabbage 15%	Cabbage 0%	5%	5%
Period 3 Cabbage 0%	Period 3 Cabbage 0%	Period 3 Cabbage 15%	Period 2 Yeast 0.2 daily Cabbage 15%	Cabbage 0%	44	50
74	68	74	96	85	37	53
49	55	79		89	38	38
Period 4 Cabbage 10%	29?	51			Period 5 Yeast 0.2 daily Cabbage 10%	Period 5 Cabbage 10%
58	Period 4 Yeast 0.2 daily Cabbage 0%	Period 4 Yeast 0.2 daily Cabbage 15%			62	66
52	73	86			Period 6 Cabbage 10%	53
Period 5 Yeast 0.2 daily Cabbage 10%	84				67	60
94	Period 5 Cabbage 10%				64	61
96	93				69	63
Period 6 Cabbage 10%	78				69	62
	80				92	
	80				Period 7 Cabbage 5%	Period 6 Cabbage 15%
					65	70
						87
						93
						Period 7 Cabbage 0.5 daily

TABLE II—*Concluded.*

Rate....4616	4639	4642	4643	4658	4639	4855
84	97				41	54
101?	92				35	46
79?	96				Period 8 Cabbage 1 daily	47
102?	Period 6 Cabbage 5%				59	32
Period 7 Cabbage 15%	82				68	Period 8 Cabbage 1 daily
96	68				61	56
103	69					65
91	Period 7 Cabbage 15%					
	92					
	91					

mental feeding. With yeast alone, even when much larger quantities were administered we have never succeeded in restoring rats which were declining on diets low in the fat-soluble vitamine.

When the ration contained only 5 per cent of dried cabbage Rats 4839 and 4855 soon declined in weight and failed to recover when provided with an abundant supply of butter fat. Since both these rats recovered very rapidly when a little yeast was added to the diet the failure to grow was obviously due to an insufficient supply of the water-soluble vitamine. Chart II shows plainly that 15 per cent of cabbage affords somewhat less than the minimum of water-soluble vitamine needed for normal growth. Expressed in terms of absolute intake the rats which grew on 15 per cent of cabbage ate 85, 86, 119, 107 gm. respectively of dried cabbage in 13 weeks; an amount not quite sufficient to supply enough water-soluble vitamine to promote growth at the normal rate. It will be noted that quantities of dried spinach somewhat smaller than those of cabbage sufficed to secure normal growth. However, one must be careful not to draw final conclusions from too few experiments, for during the first 9 weeks of experimental feeding Rat 4858 on the 5 per cent cabbage diet made as large a gain in weight as the average gain made by the rats on the 15 per cent diet although the absolute quantity of cabbage eaten by this rat was only about one-third of the average amount eaten by the rats on rations containing the larger proportion of cabbage. This is an example of the capacity of an animal to grow well, although receiving only a relatively small amount of vitamine. We have already called attention to this subject in connection with the effect of yeast as a vitamine.¹⁰

Water-Soluble Vitamine in Alfalfa, Clover, and Timothy Plants.

All these preliminary experiments with alfalfa, clover, and timothy plants in their rapidly growing state were made with too small quantities to enable us to make statements of definite value in regard to their content in water-soluble vitamine or to compare them with spinach or cabbage leaves (see Chart III). The results which were obtained with clover and timothy are similar

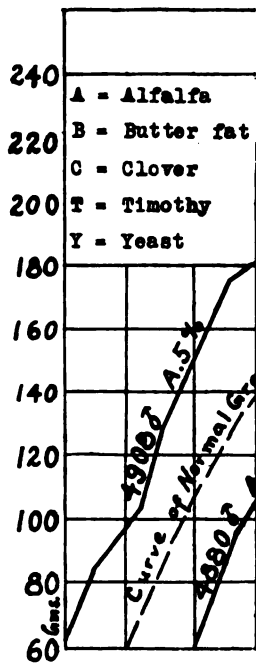
¹⁰ Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 157.

to those recorded for comparable proportions of cabbage leaves. The growth made on diets containing only 5 per cent of alfalfa indicate that this plant is somewhat richer in water-soluble vitamine than either clover or timothy. New tests, now in progress, must show the effect of feeding larger quantities of these food products than were used in these experiments.

Fat-Soluble Vitamine in Alfalfa, Clover, and Timothy Plants.

The striking feature of these experiments is the small amount of these plants needed to supply sufficient *fat-soluble* vitamine for long continued and vigorous growth. Thus in spite of the deficiency of *water-soluble* vitamine supplied in the first period all these rats at the time of writing, after 170 days of experimental feeding, are still at a weight practically normal for their age although *fat-soluble* vitamine was furnished to none of them, except Rat 4908, from any other source than the green plants. During the entire course of the experiment they have eaten only 26 and 32 gm. of alfalfa, 23, 24, and 23 gm. of clover, and 25, 21, and 20 gm. of timothy. More experiments now in progress are needed to show how much less than this small quantity will furnish enough *fat-soluble* vitamine to suffice for normal growth. We have not given the food intake of these rats fed with alfalfa, clover, or timothy, because such data have no special importance in the present discussion.

If one may draw conclusions from the limited data now available, it seems that the green vegetables supply an important addition to the diet of man because the staples such as cereals, meats, potatoes, fats, and sugar probably furnish too small an amount of either of these vitamines to meet fully the requirements of an adequate dietary. Therefore care should be taken not to reduce greatly the quantity of green vegetables customarily eaten until more is learned about the actual requirements for these food factors and their relative abundance in the commonly used vegetables and green foods. Only then will it be safe to apply the results obtained in the laboratory to attempts to effect economies in the use of these relatively expensive food products.



INFLUENCE OF HIGH TEMPERATURES AND DILUTE ALKALIES ON THE ANTINEURITIC PROPERTIES OF FOODS.

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From the standpoint of nutrition a knowledge of the stability of the water-soluble food accessory under the various conditions to which it may be subjected during the processes of food preservation and preparation is of importance. Individuals living on a mixed diet which includes fairly liberal amounts of raw fruits and vegetables without doubt are getting a considerable excess of the antineuritic vitamine; but the changing economic conditions have brought about an increased consumption of canned and dried foods. To what extent may the margin of safety be lessened by these dietary changes?

Reports of investigations dealing with the effect of high temperatures (100°C. or over) on this essential food constituent are contradictory. In certain instances the protective action against polyneuritis has been found to be destroyed in foods subjected to a temperature of 120°C. for a given length of time, while in others it apparently remains unaffected. For example, Grijns¹ found that small quantities of "Katjang-Idjo" beans (*Phaseolus radiatus*) boiled for 2 hours at 120°C. and added to peeled rice lost their power against polyneuritis in chickens. Similarly, meat, and to some extent unpeeled rice heated to 120°C. failed in their protective action. Eijkman,² however, reported that horse flesh boiled for 2 hours at this temperature did not produce polyneuritis in pigeons, although unpeeled rye, oats, millet, and barley, which had been boiled for 2 hours at 115°, 125°, and 135°C. respectively had little protective action. The antineuritic properties of wheat embryo and whey McCollum and Davis³

¹ Grijns, cited by Holst, A., *J. Hyg.*, 1907, vii, 619.

² Eijkman, C., *Virchows Arch. path. Anat.*, 1897, cxlviii, 523.

³ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

found were unimpaired after heating in an autoclave for 1 hour at 15 pounds pressure. But according to Chick and Hume⁴ wheat embryo and yeast extract lose their potency at temperatures above 100°C. Exposure for 40 minutes to a mean temperature of about 113°C. decreases the efficiency about one-half as compared with the unheated control; exposure for 2 hours to 118–124°C. reduces their power to less than one-fourth. McCollum, Simmonds, and Pitz⁵ were able to get normal growth on a ration in which navy beans, heated for 1½ hours at 15 pounds pressure (120°C.), were the sole source of the antineuritic vitamine. Comparable results were obtained by Daniels and Nichols⁶ with soy beans heated for 30 to 40 minutes at 15 pounds pressure. At 100°C. most observers have detected no destruction of the vitamine in neutral or acid media, although in dilute alkaline solutions it appears markedly affected at this temperature.

The explanation of the conflicting results in the vitamine-containing substances heated to the higher temperatures is not clear. It is possible that in certain instances small amounts of the antineuritic material were used. When the minimum amount is present a slight destruction would produce conspicuous results. It is also conceivable that the antineuritic vitamine may be present in tissues in two forms, one so bound chemically that it is unaffected by treatment which is destructive to the free or unbound form. Vedder and Williams,⁷ and Williams and Seidell⁸ have observed that acid hydrolysis of the alcoholic extract of rice polishings produced a substance more promptly curative in small doses than the original extract. The greater potency of the extract after hydrolysis may be due to the liberation of a portion which had been chemically bound. Recent studies by Cohen and Mendel⁹ relative to the etiology of scurvy suggest that in certain instances an antiscorbutic vitamine may be similarly bound. Sprouted oats and barley were found to be more effective in preventing the onset of scurvy in guinea pigs than the unsprouted seeds. It is, moreover, not improbable that the

⁴ Chick, H., and Hume, E. M., *Proc. Roy. Soc., Series B*, 1917–18, xc, 60.

⁵ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

⁶ Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

⁷ Vedder, E. B., and Williams, R. R., *Philippine J. Sc.*, B, 1913, viii, 175.

⁸ Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

⁹ Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

physiological structure of food may be a factor in determining the amount of destruction of the antineuritic vitamine, the starch and hemicelluloses of certain vegetables acting as protective material.

Studies dealing with the extracted antineuritic vitamine are of inestimable value in throwing light on the chemical nature of the complex, but from these one should not conclude that comparable changes take place in foods similarly treated. In order to know whether the observed phenomena are applicable, different types of foods which have been treated as far as possible in accordance with the household and commercial processes must be studied.

Since the usual household method of procedure consists in cooking vegetables in boiling water, while a temperature of 120°C . is most frequently used in the commercial canners and in the household pressure cookers, we have endeavored to determine whether the time required to cook certain types of vegetables under these conditions is sufficiently long to affect their antineuritic properties. Studies have also been made of the effect of cooking vegetables in dilute alkaline solutions at the boiling temperature, for the practice of adding small amounts of sodium bicarbonate (cooking soda) to the water in which such vegetables as beans, peas, and cabbage are cooked is not uncommon, in order to dissolve the intercellular material and decrease the time of cooking, or to preserve the natural color. This point is of particular interest, since the water in which foods are cooked in some sections of the country is permanently hard.

The vegetables used in the investigation were (1) cabbage, representative of the starch-free, succulent vegetable, containing small amounts of intercellular material which might, by adsorption, act as protective material to the vitamine; (2) beans, both soy and navy, foods containing considerable hemicelluloses and starch, which are most frequently cooked in the pressure cooker, or to which sodium bicarbonate is added.

In the investigation emphasis was laid on the difference in the antineuritic properties of beans cooked at high temperatures (approximately 120°C .) and those cooked in water to which dilute alkalies had been added. No comparisons were made between the cooked and raw beans since uncooked beans are not

a possible food for man. With the cabbage, studies have been made of the effect of raw cabbage and cabbage cooked under three different conditions; namely, (1) in water at 100°C., (2) in water at 120°C. for 15 minutes, and (3) in boiling water to which a slight excess of alkali had been added. The same amount of cabbage was used in all rations, the water in which the cabbage was cooked being included.

Comparable studies were made with purified rations to which the water-soluble vitamine from beans cooked at 120°C. for 20 and 40 minutes respectively were added, for it seemed probable, from the reports of other investigations, that the extracted food accessory is more sensitive to untoward influences than the unextracted material. Furthermore, it is of interest, particularly in relation to infant feeding, to know whether the liquor from vegetables similarly cooked has any antineuritic value. The possibility of using the vegetable liquor as a milk diluent suggests itself, for it has been shown by Osborne and Mendel¹⁰ that the water-soluble food accessory in milk is not so high as we have hitherto believed. The dilution of cow's milk with water or a cereal diluent low in this food accessory may result in a food containing too little of the antineuritic vitamine for physiologic well being.

Young animals show the effects of inadequate diets much more quickly than adults. This is especially true of diets lacking the water-soluble food accessory. Therefore, in our study animals (rats) from 4 to 6 weeks old were selected for the trial feedings. All the diets, as far as possible, were comparable, containing equivalent amounts of physiologically good proteins, fat, inorganic salts, and carbohydrates. Since quantitative determinations of the fat-soluble vitamine have not been possible, the diets in all cases included an excess, as shown by the results of previous investigations. In addition to the amount supplied by the vegetables used, 5 gm. of butter-fat were added to every 100 gm. of dry ration. When the ration consisted of purified materials 8 gm. were used.

There was undoubtedly some variation in the amount of water-soluble food accessory present in the several diets; but in all

¹⁰ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

cases an excess, we believe, was included. For example, 100 gm. of the navy bean ration contained 50 gm. of navy beans. Similarly, 50 gm. of beans were used as the source of the water-soluble food accessory in the purified ration, and, although the method of extraction, in all probability, did not remove completely the antineuritic vitamine, it would seem as if there were an abundant supply present, since McCollum and coworkers¹¹ have produced normal growth in rats fed rations in which the water-soluble vitamine was obtained from 25 gm. of navy beans. In our soy bean rations 55 gm. were used.

While no data are available regarding the amount of cabbage necessary to furnish sufficient water-soluble vitamine for rats, McCollum and Kennedy¹¹ conclude from their work with polyneuritic pigeons that the material is present in rather low concentration. In our rations, in order to make sure that enough was included, 250 gm. of cabbage were used to every 100 gm. of dry ration.

The extracted water-soluble material from the beans was obtained in the following manner. The beans were soaked 12 hours, then autoclaved at 15 pounds pressure for 20 minutes in the case of navy beans and 40 minutes for the soy beans. This cooked material was then comminuted by pressing through a strainer, the liquor was filtered off, and the beans were washed several times with a liberal amount of distilled water. The filtrate and washings were then evaporated to small bulk, alcohol was added to precipitate the proteins, starch, and pectins; and the material was again filtered and the alcohol distilled off.

It is obvious that by this method no conclusions can be drawn with regard to the amount of destruction that may have taken place in the food accessory at the temperature employed. We were, however, not concerned with the question of how much destruction resulted, but whether the liquor from the foods, particularly beans, thus cooked, contains appreciable quantities of the antineuritic vitamine.

Recent studies of the effect of dilute alkalies at high temperatures on the extracted antineuritic vitamine have indicated that considerable destruction takes place. McCollum and coworkers¹²

¹¹ McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

¹² McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

brought young rats to a standstill by feeding a purified ration lacking only the water-soluble vitamine. When the extract from 10 per cent of wheat embryo, which had been previously treated with a 0.28 per cent solution of sodium hydroxide at 100°C. for an hour, was added to the ration, the rats failed to grow. These authors concluded that the alkali had destroyed almost completely the antineuritic substance. In view of these results it seemed of interest to determine whether the extracted vitamine from the cooked beans was equally sensitive. Therefore in our experiments the water extracts of the beans prepared as described above were first made slightly alkaline to phenolphthalein with 0.1 N sodium hydroxide; an excess of 6.44 cc. of the sodium hydroxide solution was then added and the whole boiled in a reflux condenser for 30 and 60 minutes respectively. In all cases the water was distinctly alkaline to litmus before and after boiling.

In testing the effect of cooking vegetables with dilute alkali 33 cc. of a 5 per cent sodium bicarbonate solution were added for every 50 gm. of navy or soy beans, and 63 cc. for every 250 gm. of cabbage. The vegetables were cooked in distilled water until tender,—navy beans 90 minutes, soy bean 120 minutes, and cabbage 45 minutes. The excess alkali was neutralized with 0.1 N hydrochloric acid, and the vegetables were then combined with the other ingredients of the rations, the casein and butter fat being prepared in accordance with the usual methods.⁵

Since none of the vegetables in the amounts used supplied sufficient inorganic material for the experimental animals, one of the various salt mixtures was used in each ration to make the total inorganic content comparable to that of whole milk. A few sprinklings of ferric citrate were added to the purified rations, and two drops of a 2 per cent potassium iodide solution were added to the distilled drinking water once a week. The data concerning the composition of the various rations are given in Tables I and II.

The growth curves of the animals fed navy beans cooked at 120°C. for 20 minutes (Chart I, Group 1) as well as those fed beans cooked with an excess of sodium bicarbonate (Chart I, Group 2) indicate that in neither case was there any great destruction of the antineuritic vitamine. Growth in both groups

TABLE I.
Materials Used in 100 Gm. of Dry Ration.

Ration No.	Water-soluble food accessory.			Butter fat.	Casein.	Salt No.	Mixture.	Lard.	Cornstarch.	Filter paper.	Calories per gm.
	Source.	Amount of vegetable.	Preparation.								
		gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	
I	Navy beans.	50	Autoclaved 20 min. at 120°C.	5	8	25.18	13	18.82			4.52
II	" "	50	Boiled with 33 cc. 5% NaHCO ₃ .	5	8	25.18	13	18.82			4.52
III	Soy "	55	Autoclaved 40 min. at 120°C.	5		33.46	10	26.55			4.61
IV	" "	55	Boiled with 33 cc. 5% NaHCO ₃ .	5		33.46	10	26.55			4.61
V	Cabbage.	250	Raw.	5	15	46.53	10	63.47			4.73
VI	"	250	Boiled at 100°C.	5	15	46.53	10	63.47			4.73
VII	"	250	Autoclaved 15 min. at 120°C.	5	15	46.53	10	63.47			4.73
VIII	"	250	Boiled with 62.3 cc. 5% NaHCO ₃ .	5	15	46.53	10	63.47			4.73
IX	Soy beans.	55	Extracted from beans autoclaved 40 min. at 120°C.	8	18	18.45	10	55.55	2		4.77
X	" "	55	Same as No. IX, but extraction boiled 30 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	18.45	10	55.55	2		4.77
XI	Navy beans.	50	Extracted from beans autoclaved 20 min. at 120°C.	8	18	18.45	10	55.55	2		4.77
XII	" "	50	Same as No. XI, but extraction boiled 30 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	18.45	10	55.55	2		4.77
XIII	" "	50	Same as No. XI, but extraction boiled 60 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	18.45	10	55.55	2		4.77

TABLE II.
Composition of Salt Mixtures.

Salt mixture No.	NaCl	KCl	CaHPO ₄ 2H ₂ O	CaCl ₂	K citrate.	MgSO ₄ 7H ₂ O	Mg citrate.	Ca lactate.	Total.
1	0.876	0.3012	3.611	0.1876	2.6157	0.4255	0.4331		8.446
2	0.763		2.4191	0.4783	2.2139			0.2435	5.298
3	0.342		1.6277	0.8925	0.0201			0.5763	3.459
4	0.859	0.3868	3.355	0.0683	1.3692		0.496		6.534

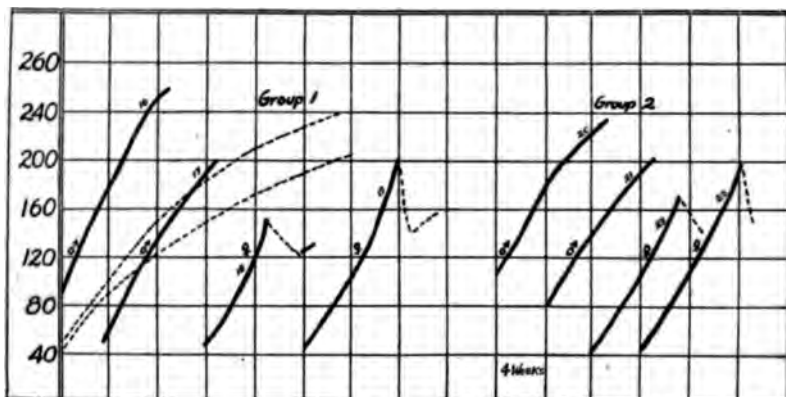


CHART I. The growth curves of animals (Group 1) fed rations in which the only source of the antineuritic vitamine was navy beans heated for 40 minutes at 120°C. (Ration I) give no indication of vitamine destruction. Nor do the curves of growth of animals (Group 2) receiving rations containing navy beans boiled in a dilute alkaline solution (Ration II) show a decrease in the potency of the vitamine.

was rather remarkable as compared with that of rats usually considered normal. Our animals on the bean rations not only grew more rapidly but reproduced at an early age.

The curves of growth of animals (Chart II) fed the rations containing the soy bean which had been treated the same way as the navy beans also indicate that neither high temperatures (Group I) nor dilute alkalies at the boiling temperature (Group 2) are very detrimental to the antineuritic vitamine in foods. As in the case of the navy beans, the growth curves surpass those usually considered normal. Similarly, reproduction occurred at an early age and the young were successfully reared.

Apparently the antineuritic vitamine of beans cooked in a pressure cooker, commercially canned beans, and beans made soft by the addition of sodium bicarbonate is not greatly affected by these processes.

The results of the feeding experiment with cabbage (Chart III) are of particular interest, since comparisons have been made between animals fed raw cabbage (Group 1) and those fed cabbage cooked in the pressure cooker (120°C.) for 15 minutes (Group 2); boiled in distilled water 45 minutes (Group 3); and

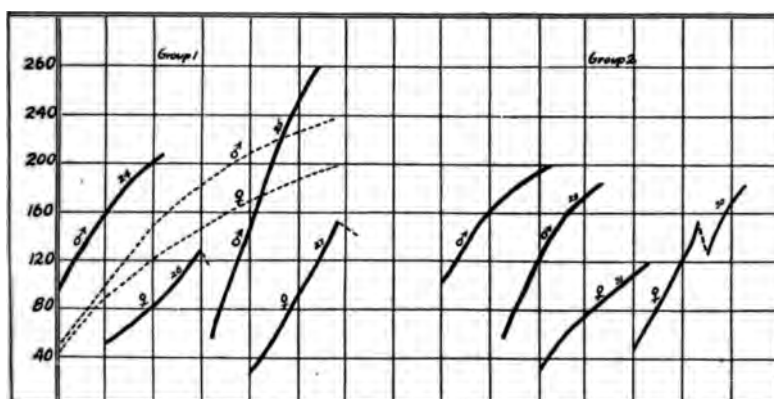


CHART II. The antineuritic vitamine of soy beans apparently is not greatly affected by cooking the beans in an autoclave at 15 pounds pressure for 40 minutes, nor by cooking at the boiling temperature in a dilute alkaline solution. The animals (Group 2) receiving the soy beans boiled with sodium bicarbonate (Ration IV) grew as well as those (Group 1) fed beans cooked at the higher temperature (Ration III).

boiled in a dilute alkali 30 minutes (Group 4). The growth curves of all are similar and give no indication that there was considerable destruction of the vitamine, resulting from the various methods of treatment. It is possible that longer cooking under similar conditions would have produced different results, but since our aim was to determine the effect on the antineuritic vitamine in vegetables prepared in accordance with the usual household and canning processes, there seemed no point in longer treatment.

Our failure to obtain results indicating that high temperatures and dilute alkalis at the boiling temperature are destructive to the antineuritic vitamine apparently was not due to the fact that a large part of the vitamine was held in the vegetable and therefore was unaffected by the method employed. The animals on the purified rations which contained the extracted water-soluble food accessory from the autoclaved soy and navy beans also made normal growth. Even the animals which received the

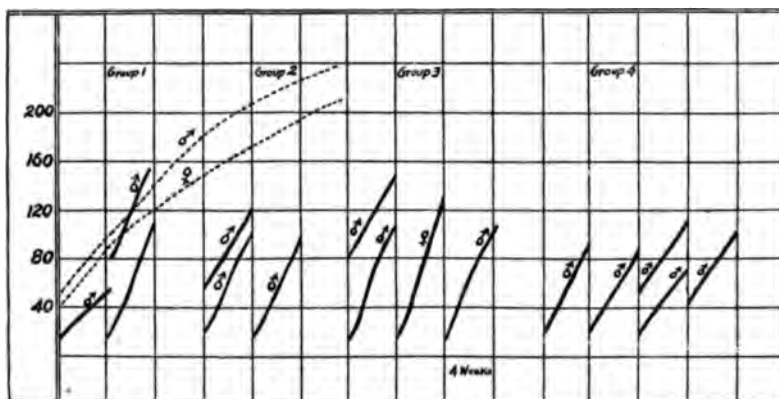


CHART III. The antineuritic vitamine of cabbage is apparently unaffected by the usual cookery processes. The animals of Group 1 received a ration in which raw cabbage (Ration V) supplied the antineuritic vitamine. Group 2 was given a similar ration which included cabbage cooked in boiling water (Ration VI). The cabbage in the ration of Group 3 was cooked at 120°C. for 15 minutes (Ration VII), while in Group 4 sodium bicarbonate was added to the water in which the cabbage was cooked (Ration VIII).

extra vitamine boiled with dilute alkali for 30 and 60 minutes respectively gave little evidence of vitamine destruction. During Period 1 (Charts IV and V, Groups 1 and 2) the failure of our animals to grow on the purified rations was undoubtedly due to an insufficient amount of the antineuritic vitamine. The rations at this time included the cold alcoholic extract from 9 per cent of raw beans. The addition of the water-soluble vitamine from a larger proportion of cooked beans stimulated growth (Periods 2). The curves during Periods 1 have been included to meet the

possible criticism that the casein supplied a sufficient amount of the vitamine to meet the needs of the animals.

Our results with the water-soluble food accessory treated with dilute alkali are not in accord with those reported by McCollum, and Simmonds.¹² Their animals receiving the water-soluble vitamine boiled for 60 minutes in an alkaline medium made prac-

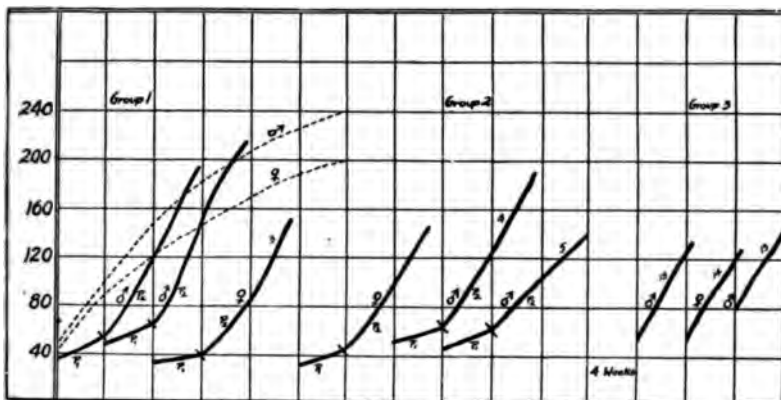


CHART IV. During Period 1, Group 1 received rations in which the water-soluble food accessory was obtained from the alcoholic extract of 9 gm. of raw navy beans, and Group 2 received a similar ration in which the food accessory had been boiled for 30 minutes with dilute alkali. An increase in the antineuritic vitamine stimulated growth in all cases. During the Periods 2, Group 1 received Ration XI containing the water-soluble vitamine from 50 gm. of navy beans, autoclaved at 15 pounds pressure for 40 minutes; Group 2 was given Ration XII, containing the same amount of the water-soluble food accessory boiled 30 minutes with a dilute alkali; and Group 2 received a similar ration (Ration XIII) containing the water-soluble material boiled 60 minutes with dilute alkali. The curves of growth suggest that the vitamine has not been materially affected by the treatment employed.

tically no growth. It is probable, however, that their rations contained the minimum amount of the antineuritic vitamine, having been obtained from 10 per cent of wheat embryo, whereas in our foods a considerable excess of the vitamine was used, and the slight destruction evidenced by the lack of growth in their animals has not been made apparent in our investigation.

The fact that our animals made normal growth on rations in which the liquor from the cooked beans was the sole source of

the water-soluble vitamine emphasizes again the undesirability of discarding the water surrounding cooked or canned vegetables. This contains not only much of the inorganic constituents of the vegetables, the soluble carbohydrates, and proteins, but, obviously, much of the water-soluble food accessory as well.

The note of warning sounded by Chick and Hume⁴ to the effect that grave danger may attend the use of large amounts of tinned foods, we believe, is unfounded, at least from the standpoint of the antineuritic vitamine content of the food. Bitting¹³

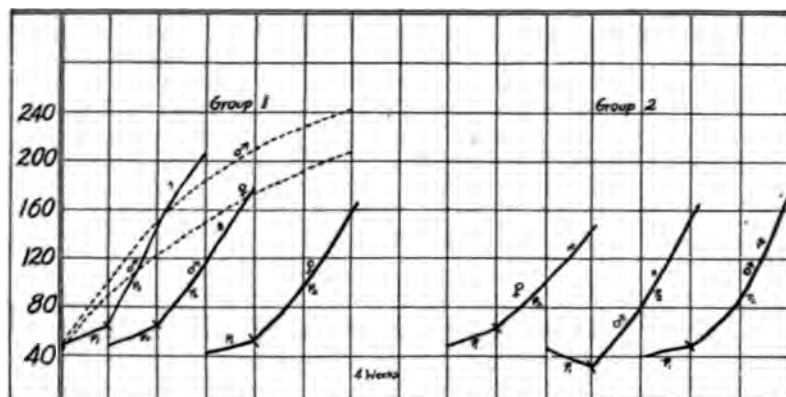


CHART V. During Period 1 the animals (Groups 1 and 2) were given rations consisting of purified foods in which the antineuritic vitamine was obtained from ground raw soy beans. The change (Period 2) to the water-soluble vitamine from 55 gm. of soy beans autoclaved at 15 pounds pressure for 40 minutes (Ration IX) produced normal growth in Group 1. Similarly, the addition of a larger amount of the water-soluble vitamine (Period 2) which had been boiled with an excess of alkali for 30 minutes (Ration X) stimulated growth in Group 2.

reports that fruits are usually processed at the boiling temperature from 12 to 15 minutes; corn at 118–120°C. for 75 to 80 minutes; green beans, lima beans, peas, and spinach at 115–118°C. for 30 to 40 minutes; and pumpkin at 120°C. for 90 minutes. From our results as well as those of McCollum and coworkers, it seems improbable that in the commercial canning of foods this vitamine is destroyed to such an extent that too little will

¹³ Bitting, A. W., *U. S. Dept. Agric., Bull.* 196, 1915.

be included in the diet where the usual amount of canned food is eaten. Even if there should be a considerable increase in the amount of canned food consumed, we believe that there will still be present enough of the antineuritic vitamine to meet the physiologic requirements, provided the diet is not materially changed in other respects. Decreasing the time required for softening beans by the use of pressure cookers or the addition of small amounts of dilute alkalies is justified, since the antineuritic vitamine of vegetables does not appear to be greatly affected by these processes.

BIOLOGICAL VALUES OF WHEAT AND ALMOND NITROGEN.

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The attempt to compare the biological values of various protein foods, made by Karl Thomas (1) in 1909, has resulted in a good deal of discussion, but few confirming experiments. The brevity of his nitrogen balance periods, as well as the arbitrary formulas used by him in the calculation of the percentage efficiencies reported, are the basis of criticism made recently by American authors (2, 3).

The comparison of minimal quantities of food nitrogen which suffice to maintain equilibrium in the adult body may, however, be accepted as indicating to some degree the relative protein values of those foods. The application of data obtained in this way may need to be carried out with caution nevertheless, since as McCollum, Simmonds, and Parsons (4) have pointed out, the nitrogen metabolism in the adult body may differ in some respects from that observed during the growth of the young, or during the process of lactation.

EXPERIMENTAL.

Two foods, wheat gluten and almond meal, were used by us in a double series of modified repetitions of nitrogen equilibrium experiments. These foods were chosen because they were thought to be relatively pure protein mixtures easily freed from the other constituents usually present in the natural foods, and might therefore be calculated as furnishing a definite quantity of absorbed amino-acid-yielding substances.

The procedure was in part that used by Steck (5), and in part that followed by Thomas (1). Six experiments were carried out,

each consisting of a fore-period of 3 days of protein-poor diet, 2 days of the protein diet, and an after-period of 1 day of protein-poor diet.

The relatively protein-free basal ration used in the fore- and after-periods, as well as with the protein in the experimental period, was of two kinds, the first rich in total carbohydrates, but poor in cellulose, the second of the same total carbohydrate con-

TABLE I.
Basal Rations.

			Nitrogen.	Calories.
		gm.	gm.	
Basal Ration I. (1st day of first gluten experiment.)	Prunes.....	100.5	0.33	303.5
	Cream.....	230.0	0.92	448.5
	Sugar.....	80.0		320.0
	Tapioca.....	115.0	0.07	411.8
	Bananas.....	242.0	0.50	239.5
	Butter.....	10.0	0.01	76.9
	Total.....		1.83	1,800.2
Basal Ration II. (3rd day of almond ex- periment.)	Swedish health bread (a rye hardtack).....	91.0	1.35	317.1
	Bananas.....	85.0	0.17	84.1
	Sugar.....	46.0		184.0
	Lettuce.....	75.0	0.12	12.0
	Olive oil.....	11.1		99.9
	Apples.....	246.0	0.15	154.8
	Celery.....	73.0	0.10	10.9
	Butter.....	75.0	0.12	576.7
	Cream.....	146.0	0.58	284.7
	Honey.....	65.8	0.04	219.4
Total.....		2.63	1,943.6	

tent, but poor in cellulose. The nitrogen content of these diets varied little as will be seen from Table I, but the effect of the difference in composition upon the availability of proteins is marked.

No attempt was made to ingest precisely the same quantity of these rations each day of the experiments, but the totals taken varied in general very little from the examples given in Table I.

The subject was a healthy young woman of 59 kilos body weight, whose weight did not change throughout the experimental period of nearly 6 months.

For the gluten experiments Sanitarium gluten sticks were used, manufactured by the Sanitarium Food Company, St. Helena, Cali-

TABLE II.
Balances with Gluten Nitrogen.

<i>Basal Diet I.</i>							
Day of experiment.	N ingested.	N excreted.			Balance.	Added N ingested per kg. body wt.	
		Urine.	Feces.	Total.			
Experiment 1.							
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		<i>gm.</i>	
1	1.83						
2	2.24	3.79	0.61	4.40	-2.16		
3	1.86	3.59	0.69	4.28	-2.42		
Gluten period.	4	6.05	5.12	0.89	6.01	+0.04	0.102
	5	6.13	5.59	0.20	5.79	+0.34	0.102
	6	1.86	4.33	—	4.33	-2.47(?)	
Experiment 2.							
1	1.60						
2	1.89	4.94	0.87	5.81	-3.92		
3	1.36	3.46	0.87	4.33	-2.97		
Gluten period.	4	4.52	3.10	0.65	3.75	+0.77	0.077
	5	4.71	5.15	0.48	5.63	-0.92	0.077
	6	1.40	2.87	0.48	3.35	-1.95	
<i>Basal Diet II.</i>							
1	2.56						
2	2.25	4.73	0.77	5.50	-3.25		
3	2.64	3.78	0.37	4.15	-1.51		
Gluten period.	4	3.98	4.80	0.35	5.15	-1.17	0.068
	5	4.07	6.11	0.33	6.44	-2.37	0.068
	6	2.28	4.50	0.33	4.83	-2.55	

fornia. The almond meal was prepared by chopping the blanched nuts very fine, sifting them, and washing five or six times with ether in order to decrease the fat content. The gluten was 6.80 per cent nitrogen; the almond meal of three different preparations, 6.28, 4.60, 4.14 per cent, respectively.

The urine and feces were collected during 5 days of the experiment, the 1st day being left out of account. Nitrogen determinations were made at once upon the fresh samples. The amount of nitrogen added in gluten or almond meal during the 2 day protein period was determined by the minimum nitrogen excretion on the preceding carbohydrate-rich diet (Tables II and V).

Biological Value of Gluten Nitrogen.

If the biological value of the gluten nitrogen is calculated from these results according to the formulas proposed by Thomas (1), the following results are obtained (Table III):

Formula A.

$$\frac{\text{Urine of N-free diet} + \text{balance}}{\text{N absorbed}} = \text{biological value.}$$

Formula B.

$$\frac{\text{Urine of N-free diet} + \text{fecal N} + \text{balance}}{\text{N ingested}} = \text{biological value.}$$

TABLE III.

Biological Value of Gluten (Wheat) Nitrogen.

Basal diet.	Experiment No.	Protein day.	Biological value.	
			A	B
I	1	1	70.5	74.8
		2	66.4	67.4
I	2	1	109.5	108.0
		2	60.2	62.1
II	1	1	72.1	74.6
		2	38.1	42.8
Average			69.4	71.6 70.5
Thomas' result with wheat flour (average) ..			37.99	42.43

If we omit from the averages for gluten the figures which strikingly deviate from the rest, those for the 1st day of the second experiment, Diet I, and the 2nd day with Diet II, the averages become 67.3 and 69.7.

The per cent of total nitrogen of the diet furnished by the gluten was as shown in Table IV.

TABLE IV.

Experiment No.	Day of experiment.	Total N of diet.	Total gluten N.	Total N from gluten.
		gm.	gm.	per cent
1 (Diet I).....	4	6.05	4.50	74.4
	5	6.12	4.50	73.5
2 (Diet I).....	4	4.52	2.98	65.9
	5	4.71	2.98	65.7
Diet II.....	4	3.98	2.27	57.0
	5	4.07	2.27	55.8
Average				65.4

TABLE V.

*Balances with Almond Nitrogen.**Basal Diet I.*

Day of experiment.	N ingested.	N excreted.			Balance.	Added N ingested per kg. body wt.
		Urine.	Feces.	Total.		

Experiment 1.

	gm.	gm.	gm.	gm.		gm.
1	1.62					
2	1.78	4.84	0.38	5.22	-3.44	
3	1.51	2.48	0.33	2.81	-1.30	
Almond 4	3.25	3.64	—	3.64	-0.39(?)	0.065
period. 5	1.36	3.95	—	3.95	-2.59(?)	

Experiment 2.

	gm.	gm.	gm.	gm.		
1	1.91					
2	1.98	4.04	0.86	4.90	-2.92	
3	1.65	3.74	1.05	3.79	-3.14	
Almond { 4	4.44	3.01	1.07	4.08	+0.36	0.071
period. { 5	3.96	4.31	1.07	5.38	-1.42	
6	1.39	4.29	1.07	5.36	-3.97	0.071

Basal Diet II.

	gm.	gm.	gm.	gm.		
1	2.12					
2	2.74	4.51	1.29	5.80	-3.06	
3	2.66	3.58	0.29	3.87	-1.21	
Almond { 4	3.53	3.63	0.50	4.13	-0.60	0.065
period. { 5	4.22	4.77	0.50	5.27	-1.05	
6	2.22	3.67	0.50	4.17	-1.95	0.065

Biological Value of Almond Nitrogen.

If we omit from the averages in Table VI the strikingly unusual figures obtained for the 2nd day of the first experiment, the averages become 86.7 and 89. This indicated high value of the nut protein is in accord with the results reported by Hoobler (6) on the value of nut proteins for human milk production.

TABLE VI.
Biological Value of Almond Nitrogen.

Basal diet.	Experiment No.	Day.	Biological value.	
			A	B
I	1	1	100.0	100.0
		2	121.3	116.6
	2	1	—	—
		2	80.2	85.6
II	1	98.3	98.3	
	2	68.2	72.0	
Average.....			93.6	94.5
			94.0	

The per cent of the total nitrogen of the diet furnished by the almond meal is shown in Table VII.

TABLE VII.

Experiment No.	Day of experiment.	Total N of diet.	Total almond N.	Total N from almond.
		<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
1 (Diet I).....	4	3.25	1.75	53.8
2 (Diet I).....	4	4.43	2.98	67.4
	5	3.96	2.98	75.2
Diet II.....	4	3.53	1.97	55.8
	5	4.21	1.97	46.8
Average				60.0

Effect of Change in Basal Diet.

It is interesting to note that the biological values of both protein foods vary considerably in the two diets. The difference can hardly be ascribed altogether to changes in per cent absorbed, due to different proportions of crude fiber in the basal ration, as will be seen from Table VIII.

TABLE VIII.
Absorption of Gluten and Almond Nitrogen.

Diet.	Experiment No.	Day.	N ingested.	N in feces.	N absorbed.	
			gm.	gm.	gm.	per cent
Gluten.						
I	1	1	6.06	0.89	5.17	85.3
		2	6.13	0.20	5.93	96.5
	2	1	4.52	0.65	3.87	85.6
		2	4.71	0.48	4.23	89.8
Average						89.3
II		1	3.98	0.35	3.63	91.0
		2	4.07	0.33	3.74	91.8
Average						91.4
Almond.						
I	2	1	4.44	1.07	3.37	78.1
		2	3.96	1.07	2.89	73.2
Average						75.6
II		1	3.53	0.50	3.03	85.9
		2	4.22	0.50	3.72	88.2
Average						87.0

Since the basal diets are not free of protein, and since the kind of protein material varies considerably in the two, a variation in its value as a supplemental source of nitrogen may well be assumed. The results recorded by Hart and Humphrey (7) in

their cattle feeding experiments with varying basal rations, indicating the efficiencies for milk production of certain protein concentrates, show similar divergences. They emphasize repeatedly the limitations of any classification of natural foods in respect to the efficiency of their proteins based on experiments involving a single food material or a single food mixture.

SUMMARY.

1. The "biological values" of wheat gluten and almond meal, determined by the usual minimum nitrogen feeding method and calculated according to the formulas proposed by Thomas, are found to average 70.5 and 94.0. This figure for wheat gluten is nearly twice that found by Thomas, using wheat flour.

2. When as much as 0.102 gm. of gluten per kilo of body weight was fed, a positive nitrogen balance was obtained, but when the intake fell to 0.077 gm. per kilo, the positive balance was not maintained, and at 0.068 gm. per kilo became negative.

3. A satisfactory positive nitrogen balance was not obtained with the largest amount of almond meal used, 0.071 gm. per kilo of body weight.

4. The character of the basal low protein diet used affected the apparent availability of these protein foods, as expressed in terms of the Thomas formulas for biological values.

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A METHOD OF EXPRESSING NUMERICALLY THE GROWTH-PROMOTING VALUE OF PROTEINS.*

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(Received for publication, December 7, 1918.)

A young animal continuously increases its food intake during growth, hence two which grow at different rates eat unlike quantities of food. We have already pointed out¹ that comparisons of the value of different proteins for growth can be made when the animals eat the same amount of food in the same number of days and gain the same amount of weight, the protein factor being the only variable. As it is exceedingly difficult to conduct experiments which fulfil these requirements we have sought to develop a method whereby the relative values of proteins for growth can be expressed numerically.

Since food intake is quite closely regulated by the calorific requirements of the animal approximately as much food is eaten under otherwise similar conditions whether this contains a high or low percentage of protein. If a single series of experiments is made with diets containing a given percentage of protein it is obviously impossible to demonstrate the *maximum* power of any protein to promote growth. When the proportion of protein in the food is so restricted that the protein factor alone determines the rate of growth, it should be possible to find the concentration which promotes the greatest gain of body weight relative to the protein ingested by supplying foods containing *different* percentages of protein. However, this is possible only within

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ Cf. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.

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limits, for differences in physical activities and the inherent capacity of the animal to grow may affect the amount of growth made within a fixed time. Consequently we cannot expect that,

TABLE I.

Source of protein.	Protein in food.	Rat.	Initial body weight.	Gain in 4 wks.	Total intake.		Intake per gm. of gain.		Gain per gm. of food.	Gain per gm. of protein.
	per cent				gm.	gm.	Food.	Protein.		
Lactalbumin.	16.2	2920♂	62	64	208	33.2	3.3	0.52	0.31	1.93
		2924♂	61	74	192	30.7	2.6	0.42	0.39	2.41
		1810♂	73	60	202	32.3	3.4	0.54	0.30	1.86
		1812♂	67	45	169	27.0	3.8	0.60	0.27	1.67
Average.....				61	198	30.8	3.3	0.52	0.32	1.97
Lactalbumin.	10.3	2474♂	71	60	213	21.9	3.6	0.37	0.28	2.74
		2931♂	63	47	189	19.5	4.0	0.42	0.25	2.41
Average.....				54	201	20.7	3.8	0.40	0.27	2.58
Lactalbumin.	7.9	2625♂	64	62	250	19.5	4.0	0.31	0.25	3.18
		2631♂	70	56	254	19.8	4.5	0.35	0.22	2.83
Average.....				59	252	19.7	4.3	0.33	0.24	3.01
Lactalbumin.	6.2	2596♂	66	31	194	12.0	6.3	0.39	0.16	2.58
		2606♂	67	39	233	14.5	6.0	0.37	0.17	2.69
Average.....				35	214	13.3	6.2	0.38	0.17	2.64
Lactalbumin.	4.9	2044♂	63	18	154	7.5	8.6	0.42	0.12	2.40
		2049♀	63	19	168	8.2	8.9	0.43	0.11	2.32
Average.....				19	161	7.9	8.8	0.43	0.12	2.36
Lactalbumin.	3.3	2201♂	68	7	131	4.3	18.7	0.62	0.05	1.63
		2203♂	67	7	172	5.7	24.6	0.82	0.04	1.23
Average.....				7	152	5.0	21.7	0.72	0.05	1.43

on a given diet, each animal will make quite the same gain of weight during equal intervals of time. How great these individual variations may be is illustrated by the data given in Tables I and II. From these we see that while the *absolute*

gains of body weight may differ by even 75 per cent, the differences between the gains in body weight *per gm. of protein eaten* are very much less, though by no means inconsiderable. It is consequently necessary to employ a larger number of animals

TABLE II.

Source of protein.	Protein in food.	Rat.	Initial body weight.	Gain in 4 wks.	Total intake.		Intake per gm. of gain.		Gain per gm. of food.	Gain per gm. of protein.
					Food.	Protein.	Food.	Protein.		
Casein.	per cent		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
	17.4	1592♂	62	54	167	29.0	3.1	0.54	0.32	1.86
		1599♂	76	49	198	34.4	4.0	0.70	0.25	1.43
		1617♂	61	74	225	39.1	3.0	0.53	0.33	1.89
		1618♂	73	63	220	38.2	3.5	0.61	0.29	1.65
		1634♂	58	53	206	35.8	3.9	0.68	0.26	1.48
		1652♂	66	59	181	31.5	3.1	0.53	0.33	1.87
		1655♂	60	43	151	26.2	3.5	0.61	0.28	1.64
		1657♂	65	55	164	28.5	3.0	0.52	0.34	1.93
	1689♂	63	50	170	29.6	3.4	0.59	0.29	1.69	
Average				55.5	187	32.5	3.4	0.59	0.30	1.74
Casein.	14.7	2465♂	77	50	203	29.8	4.1	0.60	0.25	1.68
		2619♂	64	69	238	35.0	3.5	0.51	0.29	1.97
		2620♂	65	83	246	36.2	3.0	0.44	0.34	2.29
Average				67	229	33.7	3.5	0.52	0.29	1.98
Casein.	12.0	2117♂	69	38	159	19.1	4.2	0.50	0.24	1.99
		2623♂	65	62	217	26.0	3.5	0.42	0.29	2.38
		2630♂	63	67	233	28.0	3.5	0.42	0.29	2.39
Average				56	203	24.4	3.7	0.45	0.27	2.25
Casein.	9.3	2051♂	65	19	169	15.7	8.9	0.83	0.11	1.21
		2595♂	65	29	184	17.1	6.4	0.59	0.16	1.69
		2603♂	64	27	199	18.5	7.4	0.68	0.14	1.46
Average				25	184	17.1	7.6	0.70	0.14	1.45

than were used for these experiments in order properly to minimize errors caused by such individual variations.

Table I shows that when the food contained 7.9 per cent of lactalbumin the maximum gain of body weight per gm. of protein eaten was 3.0 gm., which probably represents approximately the

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maximum growth-promoting capacity of lactalbumin when fed to albino rats under the conditions of these experiments.

Table II gives similar data obtained with casein as the sole protein of the diet. Here the greatest gain per gm. of protein eaten was 2.25 gm., the food containing 12 per cent of casein. To make this maximum gain the rats ate on the average 24.4 gm. of casein and 203 gm. of food and gained 56 gm. When the food contained 7.9 per cent of lactalbumin the rats ate on the average 19.7 gm. of lactalbumin and 252 gm. of food and gained 59 gm. Thus to make the same gain in the same time under the conditions of maximum efficiency 24 per cent more protein and 20 per cent more food were needed when the protein was casein than when it was lactalbumin.

When an animal is restricted to such a quantity of protein that a maximum gain of body weight is made per unit of protein eaten, it grows at less than the normal rate. A longer time therefore is required to make a given gain of weight, and consequently more food is needed for maintenance than if growth had not thus been delayed. In these experiments nearly the same amount of food was eaten whether this contained 6.2 or 16.2 per cent of lactalbumin (see Table I), but the gain in weight was almost twice as great on the high as on the low protein food; namely, 61 and 35 gm. respectively, or 0.32 and 0.17 gm. of gain per gm. of *food* eaten. Although growth on the 6.2 per cent ration was made with the least expenditure of *protein*, the consumption of *food* was almost twice as great per gm. of gain as that on the 16.2 per cent diet. Consequently, although an economy in the consumption of protein may be effected by reducing its concentration in the diet, this is necessarily accompanied by a larger consumption of food.

The data given in Tables I and II may be compared with those previously obtained in attempting to establish by other methods the relative value for growth of lactalbumin and casein.¹ Series A of the earlier experiments differed from those described in the present paper in that the food intake was restricted by supplying the diet in weighed quantities, estimated to be about 10 per cent less than was needed for full normal growth, and also in being continued for 11 weeks. From the data given in Table II of the earlier paper we have calculated the gains in body weight

per gm. of *protein* eaten for the entire 11 weeks, and also, for comparison with the experiments just described, for periods of 4 weeks following the time at which these rats had reached a body weight of about 70 gm. In recalculating these results we have estimated the protein from the nitrogen content of the diet whereby the nitrogen of the "protein-free milk" was included, as was done for the experiments described in this paper.

	Gain per gm. of food or protein eaten in			
	11 wks.		4 wks.	
	Food.	Protein.	Food.	Protein.
	gm.	gm.	gm.	gm.
per cent				

CORRECTION.

On page 226, Vol. XXXVII, No. 2, February, 1919, line 12, for *20 per cent more food*, read *20 per cent less food*.

periods, presumably because the former coincide with the time of most rapid growth. The maximum gains were decidedly less in these 4 week periods than those obtained in our new experiments, probably because in the earlier trials growth also was restricted by the amount of *food* supplied, and not solely by the protein eaten. When growth is limited by food intake protein can be used as a source of energy and consequently a smaller part may be available for growth than when sufficient energy is supplied in other forms and growth is determined solely by protein.

In our former paper we called attention to criticisms, based on other grounds, which might be made to Series A of the earlier experiments, which was designed to show the relative value of proteins for growth. The comparison just made gives additional reasons for rejecting such methods of experimentation.

In Chart II of our earlier paper we showed that rats made equal gains of weight in equal periods of time and ate practically

the same amount of food when diets of essentially the same composition were fed, but containing 8 per cent of lactalbumin, 12 per cent of casein, or 15 per cent of edestin. While the method employed for these experiments shows the *relative* growth-promoting power of these proteins it does not show their maximum efficiency. Thus the gain of weight per gm. of protein eaten during the 8 weeks of these experiments was for lactalbumin 2.34 gm., for casein 1.70, and for edestin 1.35 gm. These figures for lactalbumin and casein are decidedly lower than the maxima found by the method described in this paper, and the difference in the relative value of these proteins appears greater than it actually is.

For the investigator we believe that this new method, which shows with some degree of accuracy the maximum efficiency of individual proteins, or mixtures of them, for growth, will be of use. By its aid we can determine and express numerically the efficiency of combinations of proteins and compare this with that of either one alone. Differences in food intake and rate of growth are largely eliminated and experimental data can be used which cannot be compared in any other way.

This method has its limitations, for obviously when the protein of the diet is capable of promoting growth only at a very slow rate the amount of protein eaten per gm. of gain made will approach infinity as the gain approaches zero. The error thus introduced of course affects all comparisons made between figures obtained by the simple method of dividing the gain of weight by the gm. of protein eaten, but when the rate of growth on the two proteins thus compared is fairly rapid the magnitude of the error is small.

The practical feeder does not wish to know what quantity of a given protein is the smallest which he can use to secure a given amount of gain, if this quantity can only be used under unprofitable conditions. Rather does he want to know the least proportion which will give him the greatest gain in the shortest time, for although he may thereby waste some protein he may save food. The method described in our earlier paper (Series B) is better adapted to the use of the dietitian or the agriculturist in determining the relative value of proteins for growth than the method now described in this paper.

The effect of *restricted food intake* on gains made per unit of *food* eaten is shown by the following figures:

Gain per Gm. of Food Eaten.

	Protein fed.	Restricted intake.	Protein fed.	Unrestricted intake.
	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>
Lactalbumin.	16.0	0.33	16.2	0.32
	11.1	0.24	10.3	0.27
	9.2	0.20	7.9	0.24
Casein.	21.0	0.23		
	17.4	0.24	17.4	0.30
	12.0	0.19	11.0	0.27
Edestin.	9.2	0.16	9.3	0.14
	17.9	0.27	17.9	0.25
	12.4	0.16	12.4	0.20
	9.2	0.10	9.6	0.13

Comparing the gains made on diets containing similar percentages of each protein when the food intake was restricted, with those made when it was unrestricted, it is seen that with **only three exceptions** these were greater under the latter conditions of feeding. Economy of food can be effected only by supplying the young animal with as much as it will eat; economy of protein only by reducing the nutritive ratio below that at which the normal rate of growth can be maintained.

Economy in nutrition during growth depends upon a correct adjustment between the proportion of protein and the total energy supplied: furthermore, the optimum of protein is determined not only by the absolute amount furnished, but also by its quality.

NEW TITRATION METHOD FOR THE DETERMINATION OF URIC ACID IN URINE.*

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Determinations of pure uric acid have been made by permanganate titrations for many years. The presence of other substances with uric acid in body fluids, which give permanganate values, has necessitated the separation of uric acid before the titration could be carried out. Such separations have never been perfect. Whether thrown down as such by treatment with stronger acids or precipitated as urates, there has always been a part of the uric acid left in solution. Hopkins¹ came nearest the desired separation with his method of precipitation of ammonium urate. Probably the modification of Folin and Shaffer² has proved most useful of all, but it is distinctly imperfect since precipitation is incomplete even after 48 hours.

The quantitative precipitation of uric acid by adding a soluble zinc salt and then making the solution alkaline with sodium carbonate has been described in an earlier paper.³ At that time it was mentioned that Ganassini⁴ had described a qualitative test for uric acid based upon a similar precipitation. It later was

* Preliminary reports of this work were presented before the 1916 and 1917 annual meetings of the Society of Biological Chemists. Cf. Morris, J. L., *J. Biol. Chem.*, 1917, xxix, p. xiii; 1918, xxxiii, p. xxi.

¹ Hopkins, F. G., *Guy's Hosp. Rep.*, 1891, xlviii, 299.

² Folin, O., and Shaffer, P. A., *Z. physiol. Chem.*, 1901, xxxii, 552.

³ Morris, J. *Biol. Chem.*, 1916, xxv, 205.

⁴ Ganassini, D., *Boll. Soc. med. chir.*, 1908, No. 1; abstracted in *Biochem. Centr.*, 1909, viii, 250.

discovered that Bellocq⁵ knew of this reaction in 1900 and used it for determining uric acid. His method involved dissolving the precipitate in hydrochloric acid and then weighing that portion of the uric acid precipitated by hydrochloric acid. It is easy to demonstrate that all the uric acid is not precipitated by such treatment. Kashiwabara⁶ described a similar procedure for separating uric acid, then removed the zinc as sulfide before the final precipitation with hydrochloric acid. Both these methods are long and can give, at best, only approximate results. The fact, demonstrated by the writer,⁷ that uric acid can be completely and instantly precipitated as the zinc compound seemed to warrant efforts to combine this means of separation with a satisfactory method of determination. A combination with the colorimetric procedure of Folin and Denis⁷ was attempted but many difficulties were encountered. Among these the most serious was fading due to the presence of the zinc ion. The long used permanganate titration was then applied with more promising results.

A form of analysis which resulted from the combination of zinc precipitation and permanganate titration has been described before.⁸ Two preliminary precipitations for the removal of interfering substances were required before the uric acid could be separated and, though the end-point of the permanganate titration in acetic acid solution was recognizable within a few per cent, it was not entirely satisfactory. An attempt was made to improve the method by using an iodine end-point. The oxidation was carried out by using an excess of permanganate and this was followed with a larger excess of potassium iodide. It was then possible to titrate with standard thiosulfate. This method made use of the more dependable starch iodide end-point but at the cost of more time-consuming work and did not entirely eliminate the error due to spontaneous reduction of permanganate. The double titration was modified in many ways and finally was combined into one titration in which the oxidation takes place in a solution alkaline with sodium bicarbonate.⁸

⁵ Bellocq, A., *J. pharm. et chim.*, 1900, xii, 103.

⁶ Kashiwabara, M., *Z. physiol. Chem.*, 1913, lxxxiv, 223.

⁷ Folin, O., and Denis, W., *J. Biol. Chem.*, 1912-13, xiii, 469.

⁸ Morris, J. *Biol. Chem.*, 1917, xxix, p. xiii; 1918, xxxiii, p. xxi.

The use of bicarbonate was valuable for it controlled the hydrogen ion concentration so well that a mild selective oxidation took place by which all the uric acid was decomposed before any iodine was set free from the iodide. The blue starch iodide end-point could thus be used directly in the permanganate titrations. On the same principle it seemed that probably many of the substances which interfere with permanganate titrations in acid solution would be less easily oxidized than iodides and thus not interfere with the titration in sodium bicarbonate. The following substances were added to test titrations without any apparent effect: allantoin, caffeine, dextrose, hippuric acid. Phenols affected the end-point, causing a slow fading of the blue color, but earlier work³ had shown that the zinc precipitation separated uric acid quantitatively from phenols. The results from the few substances tested suggested that possibly the preliminary precipitations of interfering substances were not necessary when oxidizing in the presence of sodium bicarbonate, and investigation showed that such was substantially the case. This made it possible again to shorten the method. The final procedure, described in this paper, requires 30 to 40 minutes for the complete determination. It has proved entirely satisfactory for urine. For the much smaller quantities of uric acid in blood it gives good results also but its usefulness is limited, as is that of all other methods, by the imperfect procedures for removing proteins without at the same time carrying down other substances. When applied to the filtrates from the usual 0.01 N acetic acid precipitation the results agree with those obtained with the colorimetric method.

The steps by which the writer developed the volumetric method here described are given in detail, not only because of his belief that some light has been thrown upon the oxidation of uric acid under varying conditions but in order also to show that the idea of a titration method for determining uric acid has been consistently followed by him for more than 2 years. Curtman and Lehrman⁹ have published a volumetric method for uric acid in which the underlying principles are substantially the same as those previously set forth by the writer.⁸

⁹ Curtman, L. J., and Lehrman, A., *J. Biol. Chem.*, 1918, xxxvi, 157.

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Method.

Reagents.

Preparation of Standard Potassium Permanganate.—A stock 0.1 N solution is made up in the usual manner (3.166 gm. per liter) and kept in a tightly stoppered amber colored bottle. After the usual preliminary period, during which the value decreases somewhat, the value remains as nearly constant as its use in the preparation of 0.002 N solutions requires. When the dilute solution is needed, 10 cc. of the stock solution are measured with a pipette into a 500 cc. volumetric flask and made up to the mark. The resulting 0.002 N solution may then be standardized against a standard uric acid-phosphate solution¹⁰ by a titration such as that described later in this paper for the uric acid separated from urine. Three different 0.1 N stock solutions have upon dilution given values of 0.140, 0.143, and 0.139 mg. per 1 cc. of the 0.002 N solutions. If it is considered more convenient, the stock permanganate solution may be standardized in one of the usual ways and its equivalent value in uric acid calculated on the basis of 1 cc. of 0.1 N permanganate being equal to 7.50 mg.¹ or 7.22 mg.¹¹ of uric acid. According to our experience with the titration in bicarbonate solution 1 cc. of 0.1 N permanganate is equivalent to 7.32 mg. of uric acid. The dilute permanganate solution keeps its value for a relatively short time, but in no case have we found it to vary appreciably during a day's work.

Other Reagents Required.—Saturated disodium phosphate solution; 10 per cent zinc chloride solution; 10 per cent calcium chloride solution; 10 per cent potassium iodide solution; 20 per cent sodium carbonate solution; 0.5 per cent soluble starch solution (starch will serve but soluble starch gives a better endpoint); solid sodium bicarbonate.

Determination.

The procedure as now used is as follows: Pipette 5 cc. of urine into a 50 cc. or 100 cc. centrifuge tube and add 3 cc. of 10 per

¹⁰ Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

¹¹ von Ritter, G., *Z. physiol. Chem.* 1895-96, xxi, 290.

cent zinc chloride. Add 25 cc. of water, mix with a stirring rod, put in a small piece of litmus paper, and add enough 20 per cent sodium carbonate, with stirring, to turn the litmus entirely blue (not the violet shade of neutrality or weak alkalinity). A heavy precipitate of zinc carbonate, zinc phosphate, and zinc urate separates from a clear liquid. Now centrifugalize for 2 or 3 minutes; carefully pour off the liquid, discarding it. The precipitate is sufficiently packed down to remain in the tube. Dissolve this in dilute hydrochloric acid, with stirring. To the acid solution add 15 cc. of saturated disodium phosphate and, if necessary, a few drops more of dilute hydrochloric acid to dissolve a precipitate of zinc phosphate. Finally pour in 2 cc. of 3 per cent uranium acetate and, while stirring, again make the contents of the tube alkaline with sodium carbonate. Centrifugalize for 2 or 3 minutes and pour clear liquid carefully into a 300 to 500 cc. Erlenmeyer flask for titration. In the presence of the large excess of phosphates none of the uric acid will precipitate, while the zinc and uranium have carried down all other substances which would have given a value in the permanganate titration.

Acidify the alkaline liquid containing the uric acid with hydrochloric acid and add 25 cc. of 10 per cent calcium chloride. Then add excess of solid sodium bicarbonate. Calcium phosphate will precipitate when the acid reaction has been neutralized. An excess of sodium bicarbonate beyond this point is desirable. To the very weakly alkaline solution so prepared for titration add 1 cc. of 10 per cent potassium iodide, 3 cc. of 0.5 per cent soluble starch, and enough distilled water to make the entire volume within a few cc. of 250 cc. Now titrate with 0.002 N potassium permanganate until a blue shade spreads throughout the liquid. The color of the drop of permanganate as it hits the surface changes from pink to blue and assists in judging the approach of the end-point. Comparison of the flask from time to time with another flask containing the duplicate awaiting titration or a blank arranged for the purpose has been found to assist in arriving at the end-point. The number of cc. of standard permanganate run in from the burette needs a correction for a blank value corresponding to the amount of free iodine necessary in 250 cc. to show a perceptible blue color. This can easily be

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determined for the standard being used by titrating a blank containing 1 cc. of 10 per cent potassium iodide, 3 cc. of 0.5 per cent starch, and 250 cc. of water. It has had a value of 0.8 cc. of permanganate in our experience. Subtracting this figure from the observed reading gives the actual number of cc. of 0.002 N permanganate required for the uric acid present. Multiplying this by the value of each cc. of the permanganate gives the amount of uric acid present in 5 cc. of urine.

TABLE I.
Comparative Uric Acid Estimations.

Urine.	Benedict modification of Folin-Denis colorimetric method.	Proposed zinc precipitation-alkaline oxidation method.	Folin-Shaffer ammonium urate-acid oxidation method.
	<i>gm. per 24 hrs.</i>	<i>gm. per 24 hrs.</i>	<i>gm. per 24 hrs.</i>
F. H.*	0.592 0.586	0.592 0.592	0.527
M. S.*	0.505 0.513	0.495 0.507	0.464
Dr. C.†	0.597 0.577	0.573 0.577	
Mrs. C.‡	0.270	0.318	0.231
Mrs. X.§	0.323	0.357	0.279

* Normal.

† Nephritis.

‡ Toxic vomiting of pregnancy.

§ Pregnancy.

DISCUSSION.

It will be noted from the typical results recorded in Table I that the agreement between duplicates of the zinc precipitation-alkaline oxidation procedure is as good as those with the colorimetric method. In normal urines the values with both are practically identical. There are some irregularities in the figures given for the pathological cases. These irregularities are present in most of the pathological urines so far examined. Attempts have been made to locate the cause for this in the proposed method by the addition of such substances as might possibly be

present. The absence of any effect which can be attributed to any of the substances tried (Table II) leaves the difficulty unsolved. The figures given for the usual volumetric method (ammonium urate precipitation-acid oxidation) show that all results with that method are decidedly low.

Table II lists a few substances that might be present in urine and might, because of their nature, be suspected of affecting the titration values. The variation in titration values, with the exception of phenols, is no more in their presence than is found between separate titrations of pure solutions. Phenol gives a value in the titration and the end-point is a fading one, indicating the continued oxidation of phenol. This substance, of all those

TABLE II.

Selective Oxidation of Uric Acid by Potassium Permanganate in Solution Made Alkaline with Sodium Bicarbonate.

Uric acid standard (5 cc. = 1 mg.).	Amount of 0.002 N $KMnO_4$ required for uric acid.	Substance added.	Amount of 0.002 N $KMnO_4$ required for substance + uric acid.
cc.	cc.		cc.
5	7.0	Caffeine.	7.1
5	7.1	Allantoin.	7.0
5	7.2	Hippuric acid.	7.0
5	7.1	Dextrose.	7.2
5	7.0	Phenol.	7.8+*

* End-point indefinite and fading.

tried, most closely resembles uric acid in the selective oxidation process used. Fortunately the separation of uric acid from phenols by precipitation of the former as the zinc salt³ precludes the presence of phenols in the final titration.

SUMMARY.

A volumetric method for the determination of small amounts of uric acid has been developed and described in detail for urine. It is based upon the instantaneous and complete precipitation of uric acid as the zinc salt and a single direct titration with permanganate in a solution alkaline with sodium bicarbonate. The end-point used is the blue starch iodide color and, because of the

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mild nature of the oxidation and the excess iodide present, there is no fading to confuse the end-point.

The results on the usual blood filtrate obtained by precipitating proteins with dilute acetic acid agree with those obtained with the colorimetric method. Because of the usual difficulties which attend this preliminary precipitation of blood proteins, no detailed report has been made here of results of this application of the method. Attempts are still being made to find a precipitant of proteins which will make it possible to obtain from blood entirely reliable results with this sensitive volumetric method.

COMPARATIVE DISTRIBUTION OF UREA, CREATININE, URIC ACID, AND SUGAR IN THE BLOOD AND SPINAL FLUID.*

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In previous papers,¹ it has been shown that as the permeability of the kidney is lowered in conditions of renal insufficiency this becomes evident in the blood, first by a retention of uric acid, later by that of urea, and lastly by that of creatinine, indicating that creatinine is the most readily eliminated of these three nitrogenous waste products, and uric acid the most difficultly eliminated, with urea standing in an intermediate position. Judging from the comparative composition of the blood and urine, the kidney normally concentrates the creatinine 100 times, the urea 80 times, but the uric acid only 20 times. There is apparently little difference in the ability of the kidney to excrete creatinine and urea, although there is a marked difference between these two substances and uric acid.

In connection with these observations, it appeared of interest to us to compare the concentration of these waste products in the blood with those of the spinal fluid in cases with normal kidney activity and with nitrogen retention. With the rise in the concentration of these substances in the blood as the result of renal impairment, there must be a corresponding change in the physical factors influencing their content in the spinal fluid. Are the well known physical properties of these substances, such as solubility and diffusibility, chiefly concerned in determining

* Reported in *Proc. Soc. Exp. Biol. and Med.*, 1915-16, xiii, 126.

¹ Myers, V. C., and Fine, M. S., *Arch. Int. Med.*, 1916, xvii, 570; Chace, A. F., and Myers, V. C., *J. Am. Med. Assn.*, 1916, lxxvii, 929. Watanabe, C. K., *Am. J. Med. Sc.*, 1917, clix, 76.

how well the concentration of these substances in the spinal fluid will keep pace with the blood, or are other factors concerned?

The recent studies of Cushing and his coworkers² have given us more definite information regarding the secretion of the spinal fluid. It appears to be secreted chiefly by the choroid plexus, the cells of which are relatively impermeable to the passage from the blood stream of drugs and such substances as bile pigments. An observation made by one of us³ several years ago is of interest in this connection. Immediately after death, as soon as $\frac{1}{2}$ hour, the content of potassium salts in the spinal fluid rises to five times the normal—to the approximate concentration of the blood plasma.

It is now well known that the various membranes of the body are very permeable to urea, resulting in an even distribution⁴ of this waste product throughout the tissues of the body, and Cullen and Ellis⁵ have strikingly pointed this out in the case of the blood and spinal fluid, an observation also made by Woods⁶ at about the same time. We have likewise recorded a few observations on the spinal fluid in connection with analyses of the blood.⁷ Data on the spinal fluid of children have been reported by Leopold and Bernhard.⁸ In their normal cases they found an average urea nitrogen content of 9.9 mg. to 100 cc., 0.9 mg. of creatinine, and no uric acid, although in some of their pathological cases traces of uric acid were found. In edema fluids it has been pointed out by Denis,⁹ and also by Bernhard,¹⁰ that the concentration of the non-protein nitrogenous constituents is comparable with that of the blood.

Our own observations recorded in Table I were made during 1915 and the early part of 1916, and include estimations of the

² Cushing, H., *J. Med. Research*, 1914-15, xxxi, 1. Weed, L. H., *ibid.*, 21, 51, 93. Wegefarrth, P., *ibid.*, 119, 149. Wegefarrth and Weed, *ibid.*, 167.

³ Myers, V. C., *J. Biol. Chem.*, 1909, vi, 115.

⁴ Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

⁵ Cullen, G. E., and Ellis, A. W. M., *J. Biol. Chem.*, 1915, xx, 511.

⁶ Woods, A. C., *Arch. Int. Med.*, 1915, xvi, 577.

⁷ Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xx, 391.

⁸ Leopold, J. S., and Bernhard, A., *Am. J. Dis. Child.*, 1917, xiii, 34.

⁹ Denis, W., *Arch. Int. Med.*, 1917, xx, 879.

¹⁰ Bernhard, A., *Interstate Med. J.*, 1918, xxv, 188.

urea, creatinine, creatine, uric acid, and sugar of both the blood and spinal fluid. The estimations of the urea, creatinine, and uric acid were carried out as already described,¹¹ while the sugar was determined by a modification of the Lewis and Benedict method.¹² Creatine was determined by the method of Folin.¹³ Since these studies were completed it has been shown by several workers that the results with this method are too high. The figures for creatine have, however, been retained in our table as a matter of record. The blood and spinal fluids were obtained simultaneously, except as otherwise noted in Table I.

DISCUSSION.

Table I presents observations on blood and spinal fluid in fifteen cases. These patients were suffering from nephritis of various stages of severity, and gave chemical blood pictures varying from practically normal to excessive retention of uremia. The data are arranged according to the magnitude of the blood urea. When the blood and spinal fluid were obtained simultaneously, there was a slight lag in the urea of the spinal fluid in comparison with the blood. Averaging the whole series, the content of the spinal fluid urea amounts to 88 per cent of that of the blood. The first seven cases of the series show high figures for the creatinine of the blood, and, with one exception, the figures for the spinal fluid are likewise increased, though not in proportion to the increase found in the case of the urea. Case 5, in which the creatinine of the spinal fluid was not notably changed, was an acute and rapidly fatal case. The spinal fluid was taken 1 day previous to the blood. For the series, the creatinine of the spinal fluid represents 46 per cent of that of the blood. Apparently not more than a trace of uric acid is normally present in the spinal fluid. When sufficient fluid was available for a satisfactory estimation in these pathological cases, it was possible to obtain enough uric acid in some instances to give a fairly satisfactory color reaction. The largest amounts of uric acid were obtained in the first seven subjects, as in the case of the creati-

¹¹ Myers, V. C., and Fine, M. S., *Arch. Int. Med.*, 1916, xvii, 570.

¹² Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

¹³ Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

TABLE I.

Comparative Distribution of Urea, Creatinine, Creatine, Uric Acid, and Sugar in Blood and Spinal Fluid.

Case No.	Age.	Sex.	Urea.		Creati- nine.		Creatine.		Uric acid.		Sugar.		Remarks.
			Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	
Mg. per 100 cc.													
											per cent	per cent	
1	17	♀	288	306	14.5	4.8	11.5	1.0	12.5	0	0.19	0.09	Interstitial ne- phritis, uremia; died. Spinal fluid obtained 1 day previous to blood.
2	34	♂	208	143	11.0	4.2	5.6	0.8	9.1	0.7	0.14	—	Interstitial ne- phritis, uremia; died. Spinal fluid obtained 4 days after blood.
3	57	♂	171	—	4.8	3.9	8.9	0.5	8.0	1.5	0.14	—	Interstitial ne- phritis; im- proved.
4	3	♂	150	124	4.8	2.1	2.9	1.3	11.8	0.9	0.14	0.12	Scarlatinal ne- phritis.
5	22	♂	139	113	4.5	1.5	7.2	1.6	7.3	Trace.	0.13	0.07	Syphilis; spinal fluid taken 1 day before blood.
6	30	♂	103	105	10.8	4.0	3.6	3.1	4.7	0.5	0.16	0.09	Interstitial ne- phritis.
7	46	♂	103	96	4.8	2.1	6.4	1.3	8.6	0.9	0.27	0.12	Interstitial ne- phritis, spinal fluid taken 1 day after blood.
8	55	♂	96	96	2.3	1.8	8.6	1.0	5.7	0.1	0.19	0.09	Cheyne-Stokes respiration.
9	48	♂	90	83	2.0	1.3	4.2	1.2	5.6	0	0.15	0.11	Chronic diffuse nephritis.
10	4½	♂	83	77	3.1	1.3	4.1	1.0	5.6	0.05	0.15	0.08	Scarlatinal ne- phritis.

TABLE I—*Concluded.*

Case No.	Age.	Sex.	Urea.		Creatinine.		Creatine.		Uric acid.		Sugar.		Remarks.
			Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	Blood.	Spinal fluid.	
			Mg. per 100 cc.										
											per cent	per cent	
11	25	♀	43	41	2.6	1.4	3.7	1.2	6.4	Trace.	0.14	0.08	Chronic diffuse nephritis.
12	50	♂	39	34	2.8	1.3	3.3	1.4	3.9	0	0.14	0.08	Syphilis.
13	28	♂	32	39	2.4	1.8	—	0.9	6.3	—0.5	—	0.09	“ Spinal fluid taken 11 days after blood.
14	46	♀	26	24	2.5	1.2	2.9	1.2	3.0	—0.05	0.14	—	Gastritis.
15	71	♂	26	24	2.5	1.8	6.6	1.4	3.8	0.3	0.12	0.08	Chronic interstitial nephritis.
Average...			107	94	5.0	2.3	5.6	1.3	6.8	0.4	0.16	0.09	

nine, although there does not appear to be any close relationship between the varying amount in the blood and spinal fluid. Uric acid does not readily pass to the spinal fluid, since the amount present in this fluid represents only about 5 per cent of that found in the blood.

The comparative distribution of the sugar in the blood and spinal fluid is of interest, since the mechanism by which sugar is eliminated by the kidney is apparently quite different from that by which urea, creatinine, and uric acid are excreted. It is now generally recognized that sugar is present in the spinal fluid in amounts somewhat below that of the blood, a fact borne out by the present series of observations. Such slight differences as are present in the sugar of the spinal fluid reflect differences in the blood sugar. An observation on a case of pronounced diabetes is of interest in this connection. With a blood sugar of 0.438 per cent, the spinal fluid contained 0.30 per cent sugar. For the series, the sugar concentration of the spinal fluid amounted to about 60 per cent of the quantity present in the blood.

If the differences in concentration noted above may be regarded as representing the relative permeability of the cells separating the blood and spinal fluid, one notes that the extent of passage from the blood into the spinal fluid is greatest for urea, less for sugar, still less for creatinine, and least for uric acid. This also represents the order of their solubility in water, and apparently the order of their diffusibility through the various tissues and membranes, observations in agreement with the view of McClendon¹⁴ that the spinal fluid is an ultrafiltrate. As regards the kidney, the findings harmonize with the fact that uric acid is excreted with difficulty, but do not explain why creatinine is apparently more readily eliminated than urea. The solubility of urea is about ten times that of creatinine and its diffusibility considerably greater, although it may be noted that normally there is about fifteen times as much urea eliminated. The greater amount of work necessary for the kidney to do in the case of urea would seem to afford the only physical explanation as to why creatinine is the more readily eliminated of these two waste products.

SUMMARY.

Comparative analyses of blood and spinal fluid are reported on fifteen cases, showing varying degrees of nitrogen retention. The concentration of urea in the spinal fluid averaged 88 per cent of that of the blood, the concentration of creatinine 46 per cent, that of uric acid 5 per cent, and that of sugar 57 per cent. These percentage differences represent the order of their solubility in water, and apparently also the order of their diffusibility.

¹⁴ McClendon, J. F., *J. Am. Med. Assn.*, 1918, lxx, 977.

CREATINURIA AND ACIDOSIS.

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Boston.)

(Received for publication, December 28, 1918.)

The results of a series of experiments recently carried on in this laboratory¹ seem to prove the close connection between protein intake and the production of creatinuria in man. Briefly summarized our results indicated that in children, in two normal women, and in persons suffering from hyperthyroidism, the amount of creatine present in the urine could be increased, or decreased, at will by changes in the protein content of the diet.

It has been shown by Underhill² that in rabbits creatine excretion can be produced by the administration of a diet high in acid salts (oats and corn), and decreased to the vanishing point, either by the administration of food of an alkaline nature such as carrots, or by the use of a ration composed of an appropriate mixture of the above acid and basic foods; if, however, to this mixed ration hydrochloric acid is added, creatine excretion promptly begins, and continues as long as the administration of acid is kept up. On the basis of this experimental work Underhill has suggested the possibility of a relation between acidosis and creatine excretion.

While in our opinion the results of our feeding experiments summarized above may be considered as evidence in favor of the protein theory of creatine origin, it must be admitted that the high protein diets used by us were of a highly acid character, while our low protein diets consisted largely of alkaline vegetarian foods. It has therefore seemed undesirable to leave the subject

¹ Denis, W., *J. Biol. Chem.*, 1917, xxix, 447. Denis, W., and Kramer, J. G., *ibid.*, 1917, xxx, 189. Denis, W., and Minot, A. S., *ibid.*, 1917, xxxi, 561.

² Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

without carrying out on human subjects a few experiments along the same general lines used by Underhill in his work on rabbits.

Preliminary experiments soon showed that it was impossible to administer hydrochloric acid to human subjects in amounts sufficiently large to make our work in any way comparable to that of Underhill; we have therefore been compelled to adopt the reverse procedure, *i.e.* to feed a highly acid diet, and after creatine excretion was established to administer sodium bicarbonate in amounts sufficient to keep the urine alkaline to litmus.³

Our experimental subjects consisted of two normal boys, four women suffering from hyperthyroidism, and two normal women.

The boys and the four hyperthyroid cases were patients of this hospital; the two former were surgical cases who were being treated for fractures of the leg; they were both in good physical condition, had been subjected to no surgical procedures for 2 weeks before becoming experimental subjects, and may be considered as essentially normal.

In Experiments I to VI the subjects were given the following foods: bread, butter, eggs, milk, cheese, gelatin, one orange, and one apple. After a day or two of preliminary feeding in which the capacity of each subject was determined, a diet list composed of the above foods was made out to which the patients were required to adhere during the experimental period.

The sodium bicarbonate was administered in four equal doses at 3 hour intervals between 7 a.m. and 7 p.m.

Observations on body temperature were made at 12 hour intervals, but as no abnormal figures are recorded these data are not included in the tables.

Creatinine and creatine determinations were made by Folin's micro methods using purified picric acid; the determinations of

³ Recently Steenbock and Gross (*J. Biol. Chem.*, 1918, xxxvi, 265) have carried on a series of experiments on pigs and have shown that with these animals "protein feeding, if sufficiently intensive, will always produce creatinuria or if already present will increase it in degree. This effect obtains even during alkalosis and cannot be attributed to acids resulting from the metabolism of the protein molecule." They have further shown that administration of amounts of hydrochloric acid equivalent to the potential acidity of the ingested protein are entirely ineffective in the production of creatinuria.

hydrogen ion concentrations were made by the colorimetric technique of Henderson and Palmer.

On the whole the experimental results presented appear to demonstrate no definite connection between changes in acid-base equilibrium and creatine excretion. In Experiments I and II (normal boys), III (hyperthyroidism), and VII (normal woman) no definite change in creatine excretion was noted. In Subject 5 (hyperthyroidism) a distinct increase occurred, while in Subjects 4 and 6 (hyperthyroidism) and Subject 8 a decrease occurred.

EXPERIMENT I.

Subject 1. Male, 12 Years, Weight 31 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
<i>1918</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Feb. 23-24.....	500	0.58	0.22	6.62	0
" 24-25.....	400	0.58	0.21	6.25	0
" 25-26.....	780	0.56	0.20	9.36	10
" 26-27.....	530	0.58	0.17	10.60	10
" 27-28.....	1,660	0.51	0.17	10.29	10
" 28-Mar. 1.....	770	0.46	0.25	9.39	10
Mar. 1- 2.....	900	0.52	0.24	10.26	0
" 2- 3.....	620	0.42	0.22	7.44	0
" 3- 4.....	960	0.52	0.18	7.39	0
" 4- 5.....	—	—	—	—	0
" 5- 6.....	1,020	0.54	0.22	9.18	10
" 6- 7.....	950	0.52	0.14	9.26	10
" 7- 8.....	980	0.56	0.15	11.22	10
" 8- 9.....	1,180	0.54	0.20	10.20	10
" 9-10.....	640	0.53	0.23	8.00	0
" 10-11.....	840	0.55	0.16	9.32	0
" 11-12.....	800	0.50	0.29	9.60	0

While the experiments outlined above were in progress several papers have appeared bearing on the problem of the relation of protein intake to creatine excretion.

Rose⁴ has published an account of experiments extending over periods of several weeks, the results of which lead her to conclude that "the creatine output in women is very irregular; . . . is not definitely influenced by the amount of protein in the diet." This

⁴ Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.

series of experiments was apparently planned to furnish evidence concerning the influence of the sexual cycle on creatine excretion, and as no figures for urinary nitrogen are included comparison with our results is difficult.

EXPERIMENT II.

Subject 2. Male, 13 Years, Weight 29 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
<i>1918</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
June 9-10.....	1,400	0.84	0.21	10.64	0
" 10-11.....	1,560	0.74	0.07	6.70	0
" 11-12.....	2,000	0.94	0.16	7.50	10
" 12-13.....	1,640	0.80	0.12	6.80	10
" 13-14.....	1,860	0.76	0.15	5.95	10
" 14-15.....	1,460	0.71	0.20	5.50	10
" 15-16.....	1,280	0.70	0.16	6.08	0
" 16-17.....	940	0.71	0.12	5.82	0
" 17-18.....	890	0.79	0.19	6.49	0

EXPERIMENT III.

Subject 3. Female, Hyperthyroidism, 32 Years, Weight 45 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
<i>1918</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Feb. 2- 3.....	1,020	0.72	0.35	11.32	0
" 3- 4.....	1,400	0.70	0.56	15.89	0
" 4- 5.....	2,260	0.72	0.50	18.64	15
" 5- 6.....	1,780	0.72	0.39	14.70	15
" 6- 7.....	1,510	0.75	0.21	13.21	15
" 7- 8.....	1,400	0.75	0.65	13.89	0
" 13-14.....	1,220	0.76	0.25	11.20	0
" 14-15.....	1,020	0.76	0.24	11.30	0
" 15-16.....	1,800	0.72	0.48	14.40	15
" 16-17.....	1,480	0.69	0.23	8.65	15
" 17-18.....	1,900	0.70	0.24	10.54	15

The great variations in the daily figures for preformed creatinine contained in this work, variations in some cases amounting to 400 per cent, and the extremely low figures for preformed creatinine given for adult women, make it seem possible that some

EXPERIMENT IV.

Subject 4. Female, Hyperthyroidism, 22 Years, Weight 41 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
1918	cc.	gm.	gm.	gm.	gm.
Feb. 27-28.....	900	0.60	0.45	11.78	0
" 28-Mar. 1.....	780	0.58	0.42	11.46	0
Mar. 1-2.....	—	—	—	—	0
" 2-3.....	840	0.58	0.31	11.59	15
" 3-4.....	1,080	0.58	0.18	9.72	15
" 4-5.....	1,320	0.66	0.22	10.95	15
" 5-6.....	1,380	0.65	0.18	11.51	15
" 6-7.....	1,340	0.60	0.17	9.64	0
" 7-8.....	1,180	0.72	0.18	10.62	0

EXPERIMENT V.

Subject 5. Female, Hyperthyroidism, 23 Years, Weight 51 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	pH	Sodium bicarbonate taken.
1918	cc.	gm.	gm.	gm.		gm.
Apr. 27-28.....	1,160	0.50	0.43	11.60	5.1	0
" 28-29.....	1,140	0.50	0.44	11.70	5.3	0
" 29-30.....	1,160	0.46	0.43	12.08	5.3	10
" 30-May 1.....	1,250	0.62	0.65	15.8	7.0	15
May 1-2.....	1,240	0.49	0.40	10.29	7.0	15
" 2-3.....	1,600	0.56	0.44	12.64	7.0	15
" 3-4.....	1,150	0.61	0.38	10.69	7.0	0

EXPERIMENT VI.

Subject 6. Female, Hyperthyroidism, 27 Years, Weight 63.3 Kilos.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	pH	Sodium bicarbonate taken.
1918	cc.	gm.	gm.	gm.		gm.
Apr. 28-29.....	1,140	0.70	0.88	19.72	5.1	0
" 29-30.....	1,040	0.73	0.94	20.59	5.7	10
" 30-May 1.....	1,380	0.60	0.55	13.66	6.3	15
May 1-2.....	2,140	0.81	0.49	19.68	7.0	15
" 2-3.....	1,720	0.67	0.55	17.71	7.0	15
" 3-4.....	1,600	0.81	0.32	15.20	6.2	0
" 4-5.....	1,360	0.68	0.65	15.09	5.0	0
" 5-6.....	1,440	0.80	0.88	18.14	5.0	0
" 6-7.....	1,280	0.66	0.66	16.00	5.3	0

of the experimental subjects could scarcely be classed as normal. For instance in Experiment I a figure of 85 mg. of preformed creatinine is given as the amount of this substance excreted on February 23, 1915, by a woman weighing 60 kilos. 6 days later, March 1, the same subject excreted 162 mg. of preformed creatine, and on March 4, 356 mg. As a common figure for preformed creatinine excretion in 24 hours for infants of 1 year weighing about 10 kilos is in the neighborhood of 100 mg., it is difficult to connect some of the above figures with normal adult women.

EXPERIMENT VII.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
1918	cc.	gm.	gm.	gm.	gm.
Jan. 21-22.....	1,220	1.14	0	6.0	0
" 22-23.....	1,060	1.16	0.12	—	0
" 23-24.....	1,160	1.15	0.07	—	0
" 24-25.....	1,180	1.21	0.18	—	0
" 25-26.....	960	1.03	0.32	13.49	0
" 26-27.....	1,280	1.23	0.59	21.63	0
" 27-28.....	1,060	1.26	0.38	17.28	10
" 28-29.....	960	1.07	0.27	17.37	15
" 29-30.....	1,080	1.18	0.41	19.98	15
" 30-31.....	1,500	1.57	0.30	21.75	15

Subject 7 --Normal female, 39 years, weight 90 kilos. Jan. 21 to 22. a low protein diet free from meat was taken. On Jan. 22, a high protein diet consisting of the following foods was started and continued to the end of the experiment: bread 200 gm., butter 50 gm., twelve eggs, gelatin 50 gm., cheese 200 gm., two oranges, two lemons. This diet contains approximately 29 gm. of nitrogen and 2,200 calories.

Rose, Dimmitt, and Bartlett⁵ have also published the results of experiments on the subject of high protein feeding which have caused them to come to the conclusion that "the ingestion of diets excessively high in protein fails to induce the excretion of creatine in normal women and men." In connection with this conclusion we would call attention to the fact that the two experiments on women made by these investigators are not in any way a repetition of our experiments on this subject. Thus in their

⁵ Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918. xxxiv, 601.

experiments the highest diet taken by the female subject A.B. contained 21.76 gm. of nitrogen and 2,243 calories per day, while the female subject E. B. took only 16.16 gm. of nitrogen and 1,759 calories; furthermore, these diets were taken for a period of 5 days only and followed a period of in one case 6 and the other 5 days during which a low protein diet had been consumed.

EXPERIMENT VIII.

Date.	24 hour volume.	Creatinine.	Creatine.	Total nitrogen.	Sodium bicarbonate taken.
1918	cc.	gm.	gm.	gm.	gm.
Jan. 17-18.....	1,020	1.19	0	5.2	0
" 18-19.....	1,220	1.13	0	—	0
" 19-20.....	1,060	1.16	0.08	—	0
" 20-21.....	980	0.99	0.06	—	0
" 21-22.....	1,020	1.10	0	—	0
" 22-23.....	1,200	1.19	0.17	—	0
" 23-24.....					
" 24-25.....	1,080	1.08	0.20	16.60	0
" 25-26.....	1,030	1.00	0.28	17.32	0
" 26-27.....	1,110	1.22	0.38	21.78	0
" 27-28.....	1,300	1.01	0.30	23.63	0
" 28-29.....	1,320	1.13	0.11	23.49	10
" 29-30.....	1,120	1.11	0.14	20.49	15
" 30-31.....	1,160	1.16	0.11	19.25	15
" 31-Feb. 1.....	1,170	0.99	0.12	18.25	15
Feb. 1-2.....	1,120	1.12	0.18	20.16	0
" 2-3.....	1,540	1.05	0.15	21.16	0

Subject 8.—Normal female, 23 years, weight, 53 kilos. Jan. 17 to 18 was the last day of an 8 day period of low protein feeding. On Jan. 18, a period of high protein feeding was started and continued to the end of the experiment. The daily ration consisted of the following: bread 150 gm., butter 25 gm., milk 500 cc., twelve eggs, gelatin 55 gm., cheese 200 gm., string beans (canned) 100 gm. This diet contains approximately 31 gm. of nitrogen and 2,200 calories.

In the experiments which justified our statement that creatinuria could be produced in women by high protein feeding, the daily ration contained approximately 33 gm. of nitrogen, an amount a little more than twice that contained in the diet of the second female subject of Rose, Dimmitt, and Bartlett. Furthermore, we have found by experience, that in the production of

creatinuria by forced protein feeding, the desired result is obtained more slowly if the preceding diet has been of a low protein nature.

The two normal women who were used in our experiments of last year were again used in Experiments VII and VIII of this paper, and as will be seen it took several days of high protein feeding before creatinuria was induced. It is to be admitted that more experimental work on a larger number of women is necessary before a decision can be reached in this matter. It is, however, extremely difficult to obtain female subjects possessing sufficient physical courage and scientific enthusiasm to carry them through such a disagreeable dietary experiment, and, as after a considerable number of trials, we have been unable to obtain further experimental material our first paper was published in the hope that our experiments might stimulate sufficient interest to cause them to be repeated in other laboratories.

THE ANTISCORBUTIC PROPERTY OF VEGETABLES.

I. AN EXPERIMENTAL STUDY OF RAW AND DRIED TOMATOES.*

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(Received for publication, December 18, 1918.)

The following experiments are a part of a large series planned to determine the antiscorbutic potency of different foods, the effect of preparation and preservation upon these foods, and other similar questions. Desiccation of certain foods, particularly vegetables, as a means of preservation, has been advocated widely because of war conditions. There can be no question but that such a procedure is justified from an economic standpoint, but concerning the physiological value of desiccated foods we need enlightenment. Givens and Cohen¹ have reported an experimental study of dried cabbage. This work on dried vegetables we have continued at the request of the Division of Food and Nutrition, Medical Department, U. S. Army. It is of great importance to have all possible information concerning foods accessible for incorporation into the dietary of our soldiers and civilians.

There has been a difference of opinion as to the existence of a third type of vitamine, one protecting against scurvy. From the work of Holst and Frölich,² Chick and Hume,³ Hess and Unger,⁴ Harden and Zilva,⁵ Cohen and Mendel,⁶ and Givens and

* A preliminary report of this work was given at the meeting of the Society for Experimental Biology and Medicine, Oct. 16, 1918.

¹ Givens, M. H., and Cohen, B., *J. Biol. Chem.*, 1918, xxxvi, 127.

² Holst, A., and Frölich, T., *Z. Hyg.*, 1912, lxxii, 1; 1913, lxxv, 334.

³ Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1916-17, x, 141.

⁴ Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 479, 487.

⁵ Harden, A., and Zilva, S. S., *J. Inst. Brewing*, 1918, xxiv, 197; *Biochem. J.*, 1918, xii, 93.

⁶ Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

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Cohen,¹ there is evidence of the presence in certain foods of some substance which behaves like a vitamine by affording protection against experimental scurvy in the guinea pig.

It is widely recognized that scurvy can be prevented or cured by the consumption of fresh vegetables. To locate citations directing the use of specific vegetables and experimental evidence for that use is another question. We are able to find only one direct statement bearing on the use of raw tomatoes as antiscorbutic material. Munson⁷ states that "the raw potato, onion, and tomato are especially valuable" in the prophylaxis of scurvy. The tomato has long enjoyed the status of an unessential accessory article of diet, its value being attributed to its flavor. That it can play the rôle of an essential accessory in preventing the development of scurvy, we believe the following experiments will show.

There has not been an extensive use of dried vegetables as antiscorbutic agents. Presumably the reasons for this are twofold. In the first place, until lately only a few dehydrated foods for human consumption have been on the market. Secondly, notwithstanding propaganda to stimulate the use of desiccated foods, particularly green stuffs, there has been offered very little scientific evidence guaranteeing the presence in the dried materials of the original properties of the raw. In fact, the majority of such evidence which has been offered regarding the antiscorbutic potency of dried foods has been of a negative character.^{2, 3} If it can be physiologically demonstrated that desiccated foods retain all their original properties, then the case of preservation by dehydration is complete and justified.

Holst and Frölich,² and Chick and Hume³ found dried vegetables more or less deficient in the antiscorbutic property. Recently Chick, Hume, and Skelton³ have reported, with other interesting data, that the feeble antiscorbutic property of fresh milk is destroyed either by drying or the long keeping after drying. Hess and Unger⁴ have stated that "dehydrated vegetables were found to contain little or no antiscorbutic virtue."

⁷ Munson, E. L., *The theory and practice of military hygiene*, New York, 1901, 762.

³ Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

They draw this conclusion from the fact that in certain experiments guinea pigs were not protected from scurvy by eating dried carrots. Stefánsson⁹ alleges that "the strongest antiscorbutic qualities reside in certain fresh foods and diminish or disappear with storage by any of the common methods of preservation—canning, pickling, drying, etc." His statement is based upon his experience with such products used as rations for men on polar expeditions.

Givens and Cohen¹ have reported experiments dealing with the influence of temperature on the antiscorbutic vitamine in the drying of cabbage. It is their opinion, as regards this vegetable, that the degree of temperature in drying is the factor which determines the absence or presence of the accessory scurvy-protecting substance. Most of their dried material was used within a month after its preparation, consequently no evidence was offered as to the effect of aging. This point will be reported upon in the future. In the following experiments the influence of temperature on the antiscorbutic property of the tomato is shown. The selected temperatures at which the drying was carried out also permit certain conclusions regarding the effect of length of time of drying. The problem of the potato is more complicated than is indicated by the report of Givens and Cohen,¹ consequently it will be dealt with in detail in a future communication.

Drier and Drying of Tomatoes.

Because of the lack of available, accurate information on the best mode and temperature for drying foods, we constructed a special apparatus.¹⁰ Accordingly a drier has been manufactured which permits us to control the temperature within 5° or less, at all times. The volume of air is always the same. The humidity of the entering air depends on atmospheric conditions. However, the ingoing air can be dried if such a procedure appears necessary.

The apparatus is made of galvanized metal and is covered with asbestos. In the sides are drawers, having framework only for

⁹ Stefánsson, V., *J. Am. Med. Assn.*, 1918, lxxi, 1715.

¹⁰ Our thanks are due Mr. J. F. Barker, President of Mechanics Institute, Rochester, for designing and directing the construction of the drier.

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their sides and with the bottoms made of wire netting, $\frac{1}{2}$ inch mesh. Drawers so constructed permit a free circulation of air through all parts. In the front end of the machine is located a 12 inch fan. Midway between the fan and the first section of drawers a number of electric heating coils are placed. The entering air is thus heated before it strikes the first section of drawers. After each section of drawers there are supplementary electric coils. These coils bring the moving air, which has been slightly cooled as it passed over the first section, up to its former temperature. A thermostat controls the heating current. The temperature of each drawer can be determined at any time by the two thermometers inserted. The details of the drier are shown in Fig. 1.

Tomatoes dried at two ranges of temperature have been used; "low-dried," 35–40°C., and "high-dried," 55–60°C. The maximum and minimum times of drying for the "low-dried" tomatoes were 52 and 35 hours respectively; for the "high-dried," 26 and 13 hours respectively. The variations in the time of drying are caused partly by the difference in water content of various samples of tomato and partly by the variable moisture content of the air. None of the tomatoes were dried without interruption. The process had to be stopped at the end of each day and was taken up on the next.¹¹

The tomatoes were wiped clean with a cloth, stems removed, and bad spots, if any, excised. They were then cut into pieces shaped like a pyramid, the skin forming the base, and placed so on the mesh bottom drawers of the drier. A slight amount of juice was lost in the cutting, but since the operation was done in pans, the juice was weighed, and corresponding corrections made for the percentage of the dried product in terms of the raw. The tomatoes were kept in the drier until there was no further loss in weight in the course of an hour's drying. The dried products were kept in ordinary glass-capped, rubber-ringed preserving jars. Details concerning the dehydrated products, with the time elapsing between drying and using, are given in Table I.

The high-dried product differed from the low-dried in that it was subjected to 20°C. higher temperature and the time of drying

¹¹ We are indebted to Misses Van Horne and Venor, of Mechanics Institute, for drying under our direction the materials used in these experiments.

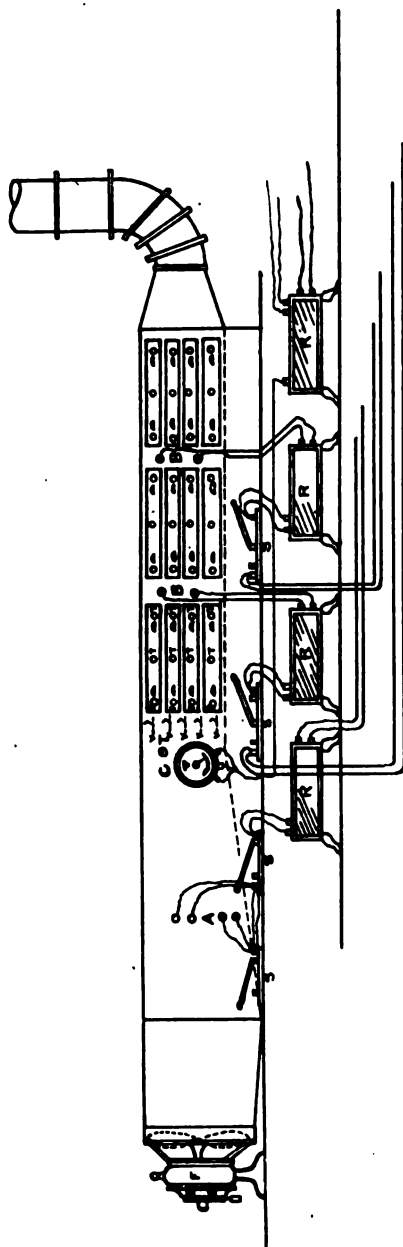


FIG. 1. Sketch of drier. F, fan; A, heating coils; C, thermostatic control; B, supplementary electric coils; T, thermometers; R, resistance for control of heat; S, switches; V, vanes.

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reduced. Also it contained slightly less water. All samples of dried tomatoes were fed as such, the daily allowance of this supplement being 1 gm. per animal. The animals ate greedily this allotment.

Raw tomatoes were always given in the amount of 10 gm. per animal per day. They were wiped off with a damp cloth and sliced so that each animal received approximately the same proportion of skin, pulp, and seed. Guinea pigs relish raw tomatoes.

TABLE 1.

Lot No.	Sample No.	Date dried.	Date started using.	Date finished using.	Duration of drying.	Per cent of original weight.
Low-dried tomatoes, 35-40°C.						
		1918	1918	1918	hrs.	
XXIII	1, 2	Aug. 15	Aug. 21	Sept. 13	36½	6-7
31	1	" 23	Sept. 13	Oct. 3	35½	6.5
34	2	" 29	Oct. 4	" 16	38½	7.3
31	2	" 23	" 17	" 29	35½	6.7
37	1	" 30	" 29	Nov. 7	42½	7.5
34	3	" 29	Nov. 8	" 18	38½	7.3
High-dried tomatoes, 55-60°C.						
XVIII	2	July 31	Aug. 1	Aug. 24	23	6.5
	1	" 31	" 25	Sept. 25	23	6.5
XX	1	Aug. 3	Sept. 26	Oct. 12	14	6.5
	2	" 3	Oct. 13	" 26	14	6.5
XI	1	" 7	" 27	Nov. 1	14½	7.1
XXII	—	" 8	Nov. 2	" 5	14	7.2
38	3	Sept. 12	" 6	" 16	24½	6.8
39	1	" 13	" 17	" 18	25½	7.3

Diet.

The basal diet was a dried product containing soy bean flour, milk, yeast, paper pulp, sodium chloride, and calcium lactate. It was made according to the directions given by Givens and Cohen.¹ In certain cases more of the heated soy flour has been added to the diet; the specific instances are noted in Table II.

In the light of our present knowledge of the so called biological properties of foods, based on experiments conducted on the rat, the diet of oats alone used to produce scurvy in guinea pigs can

be criticised. McCollum and Pitz¹² allege that oats are deficient in adequate proteins, inorganic salts, and their fat-soluble A. If we accept their standard for a complete diet, then the experiments of Cohen and Mendel,⁶ of Givens and Cohen,¹ and the present work, on scurvy produced experimentally in the guinea pig on a soy bean mixture, are the only ones in which the diet was complete. This diet contains adequate protein, sufficient fat, carbohydrates, and inorganic salts, and a liberal supply of fat- and water-soluble vitamins. This statement is based on the fact that Givens and Cohen were able to induce normal growth in rats supplied this diet *ad libitum*. However, these experiments are open to the criticism that in supplying the diet *ad libitum* to the rats, they obtained more of the vitamins than would have been the case if the food intake had been limited as it was with the guinea pigs. That such a criticism is not valid we believe from the following experiments conducted on pigeons.

Two pigeons have been fed on the same basal soy cake mixture which produces scurvy in the guinea pig. Their daily supply of this food has been limited to the same weighed amount received daily by the guinea pigs. The actual daily intake, unless otherwise noted, of the guinea pigs and the birds was approximately 11.1 gm. of soy flour, 14.0 cc. of whole milk, 0.44 gm. of dried yeast, 1.1 gm. of filter paper, 0.44 gm. of calcium lactate, and 0.44 gm. of sodium chloride. The birds ate very little of the food mixture at the beginning of the experiment. Beginning with the 16th day and continuously since, they have consumed all the food allotted per day. These birds are now perfectly healthy after receiving the soy cake mixture for 105 days (Fig. 2). They have never shown any signs of polyneuritis. That they should drop in weight at first and not regain at once their initial weight was to be expected when they did not eat, and when the nutrients fed were limited and no doubt insufficient in amount to meet their metabolic requirements. However, the experiments confirm the results obtained on feeding the soy cake mixture to rats, in that they demonstrate conclusively that the soy cake mixture contains a sufficient amount of the water-soluble vitamins to

¹² McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

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prevent polyneuritis in the pigeon—a species particularly susceptible to this disease. That the diet contains enough of the fat-soluble vitamine also we believe has been demonstrated by the work of Cohen and Mendel and of Givens and Cohen. This assumption is further supported by the results reported by Osborne and Mendel¹³ and by Daniels and Nichols.¹⁴ In the soy cake mixture used in these experiments, the amount of fat-soluble vitamine in the soy flour has been augmented by the addition of whole milk. Bulk in the diet has been provided in the form of paper pulp. The inorganic constituents have been supplemented by the addition of sodium chloride and calcium lactate. In the light of the present available evidence, we feel justified in concluding that the diet used to produce scurvy in guinea pigs in these experiments is adequate in every respect, with the one exception of being deficient in a vitamine protecting against scurvy. Such a diet removes the possibility of the influence of any deficiencies other than the antiscorbutic vitamine.

General Procedure and Methods.

All the guinea pigs used were young ones. In general we tried to obtain them at about 8 weeks of age. They were the offspring of strong, healthy stock. Reliable parties conducted the breeding and rearing of the animals and kept us informed regarding the manner in which they were handled. The food consumed by the guinea pigs prior to starting the experimental diet was varied, and contained plenty of green foods. We mention these facts because a properly reared animal is much stronger and more healthy than one treated in a haphazard way.

When an experiment was started, each animal was placed in a separate metal cage and kept there alone at all times. Once daily, weighed amounts of food were supplied in individual cups. Tap water was given *ad libitum*. The animals were examined every day and weighed every other day. In this way the first appearance of the specific symptoms of scurvy were noted.

Autopsies were performed as early as possible after death. Animals in a moribund condition were usually anesthetized and

¹³ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 369.

¹⁴ Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

examined at once. A decision of scurvy was based upon clinical manifestations and the usual autopsy findings (Givens and Cohen,¹ Chick, Hume, and Skelton²). Of course, when curative measures were instituted and the animal saved, scurvy was diagnosed from the usual external symptoms and that diagnosis confirmed by autopsies on control animals. The results are shown in Table II and Figs. 3 to 6.

Results.

For control experiments on the soy cake mixture and this mixture plus a small supplement of green food, as cabbage, reference is made to the charts of Givens and Cohen. These workers found that guinea pigs on the soy cake mixture *alone* regularly developed scurvy and succumbed in 16 to 26 days unless curative measures were instituted.

Experiments with Raw and Dried Tomatoes.

In the present experiments raw tomatoes have been used both as a preventive and curative agent in experimental scurvy of the guinea pig. Group 1 (Table II and Fig. 3) demonstrates conclusively that a daily supplement of 10 gm. of raw tomatoes will afford a guinea pig protection against scurvy when that animal is on a diet known to produce the disease in this species. These animals have grown and been maintained in apparently perfect health for a period of 110 days. If the supplement of raw tomatoes had not been given, these animals, as has been proved, would have died of scurvy in 16 to 26 days. That the tomato will serve equally well as a curative is shown by Group 2 (Table II and Fig. 4). These animals developed signs of scurvy in 18 to 19 days. After a few days, each guinea pig was fed daily a supplement of 10 gm. of raw tomatoes. They responded to the treatment in a few days, became lively, their symptoms of scurvy gradually disappeared, and they began to increase in weight. They are now, at 100 days, large, healthy animals. In Guinea Pig 227 (Fig. 3), which died on the 31st day of the experiment, no macroscopic signs of scurvy were present.

Group 3 (Table II and Fig. 5) represents a series of experiments which demonstrate that tomatoes dried at a low temperature

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TABLE II.

Group and diet.	No.	Weight.			Duration of experiment.	Remarks.
		Initial.	Maximum	Final.		
		gm.	gm.	gm.	days	
1 Soy diet + 10 gm. raw tomatoes daily.	227	157	225	192*	30	Cause of death unknown. No signs of scurvy.
	228	150	374	374	110	Never any signs of scurvy.
	229	199	410	410	110	" " " " "
	239	160	390	390	90	" " " " "
2 Soy diet alone until scorbutic; then daily supplement of 10 gm. raw tomatoes.	237	142	364	364	100	Symptoms appeared on 19th day. Supplement of 10 gm. raw tomatoes daily beginning on 20th day. Cured of scurvy.
	238	148	322	322	100	Symptoms appeared on 15th day. Supplement of 10 gm. raw tomatoes daily beginning on 18th day. Cured of scurvy.
3 Soy diet + 1 gm. of low-dried (35-40°) tomatoes raw.	240	106	280	270*	74	Cause of death anatomical anomaly. No signs of scurvy.
	241	167	309	290	92	Never any signs of scurvy.
	242	137	230	198*	72	Cause of death unknown. No signs of scurvy.
	243	136	308	296	92	Never any signs of scurvy.
	244	172	320	320	92	" " " " "
4 Soy diet + 1 gm. high-dried (55-60°) tomatoes raw.	230	164	374	374	110	Never any signs of scurvy.
	231	176	388	388	110	" " " " "
	232	172	436	436	110	" " " " "

* Weight at death.

(35–40°C.) retain some of the original antiscorbutic potency of the raw tomato. A supplement of 1 gm. daily of this dried material has been effective in protecting these animals for 92 days.

Group 4 (Table II and Fig. 6) shows that tomatoes dried at a temperature of 55–60°C. also retain some of the antiscorbutic vitamine. A daily supplement of 1 gm. of this material has afforded these animals protection against scurvy for 110 days.

DISCUSSION.

It has been established that a diet apparently adequate for the rat and the pigeon will produce scurvy in the guinea pig. If this diet is supplemented with a daily allowance of 10 gm. of raw tomatoes, no signs of scurvy will develop in the latter species. This property of raw tomato is also effective as a curative agent, yielding results similar to those obtained with raw orange juice. Therefore it seems demonstrated that the tomato may be considered to contain some of the essential dietary accessory substance.

Any discussion of the results obtained with dried tomatoes must be governed by the experimental procedures employed. Only one kind of drying has been employed—hot air. The temperatures at which the products were dried differed by a range of 20°C., consequently there is a difference in the duration of drying. It might be conceived that a longer duration of drying at a low temperature was more destructive than a short duration at a high temperature. No light is thrown upon this point in the present experiments because it has not been established what minimum amount is necessary of either the low- or high-dried materials barely to protect a guinea pig against scurvy.

The dried products used here permit of some consideration of the possible influence of aging. The low-dried tomatoes were still effective as antiscorbutic agents 80 days after drying; the high-dried, 90 days. This fact, however, does not preclude the possibility of some destruction or spontaneous disintegration of the antiscorbutic vitamine. Until there has been worked out a method for determining quantitatively the amount of this vitamine in a food, little that is definite can be said regarding the effect of aging in a dried product which retains some of its antiscurvy vitamine. It might be assumed that there was contained in 1

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gm. of the dried tomatoes an excess of the accessory above the amount necessary to protect the given body weight of animals used. Then this excess could have been destroyed in the aging and still we should have encountered no signs of scurvy. At any rate it is logical to conclude that tomatoes dried in the manner here described retain some of their antiscorbutic potency after storage of 3 months.

Pitz¹⁵ has offered experiments to show that sodium chloride and calcium lactate afforded guinea pigs some protection against scurvy, and therefore delayed the appearance of scorbutic signs. Our basal diet contained more of both of these salts than the one he used. We obtained scurvy in our animals in a very short time, indicating no favorable influence of the salts when present in the soy flour-milk-yeast-paper mixture. However, we concur in the opinion expressed by Hess and Unger that it is impossible to interpret contradictory results when dissimilar basal dietaries are used. The results with low-dried tomatoes confirm those obtained by Givens and Cohen with cabbage dried at a low temperature. No comparison can be made between the findings with high-dried cabbage and high-dried tomatoes. The former were dehydrated at a higher temperature and were subjected to a different kind of heat; *viz.*, moist heat. Holst and Frölich report that the antiscorbutic substance in food is very easily destroyed by high temperatures with moisture present.

SUMMARY.

Experiments have been carried out which show that the raw fresh tomato is a very efficient antiscorbutic agent. A small daily addition of this substance to a diet known to produce scurvy in guinea pigs maintained these animals in apparently perfect health. It is here demonstrated that raw fresh tomatoes dried in a blast of air at either a low temperature (35–40°C.) or a high temperature (55–60°C.) retain a *significant amount* of their antiscorbutic potency. It is possible that the drying at either temperature may destroy some of the antiscorbutic vitamines but to what degree cannot be stated yet. A daily supplement of 1 gm. of raw dried tomatoes protected guinea pigs from experimental scurvy.

¹⁵ Pitz, W., *J. Biol. Chem.*, 1918, xxxvi, 439.

These experiments lend confirmation to the results of Chick, Hume, and Skelton who believe "that guinea-pig scurvy is due to the deficiency in the diet of a specific accessory substance." They support the contention of Cohen and Mendel that roughage, as it affects the texture of the diet, is not the determining factor.

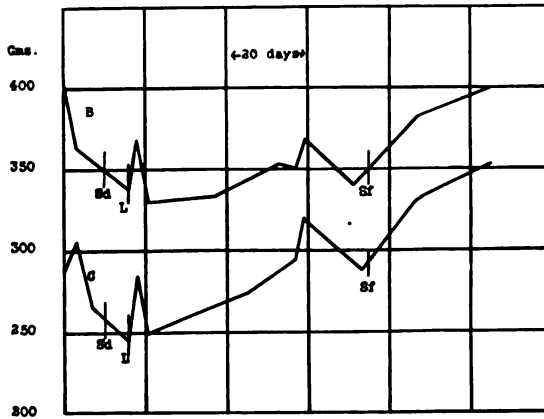


FIG. 2. Curves of body weights of two pigeons fed on the basal scurvy-producing diet. The curve of Pigeon B is moved 50 gm. above actual weights to keep it from cutting the other curve. From the 10th day on the birds were supplied with sand and gravel (Sd). At first the pigeons did not eat all of the soy cake mixture. By the 16th day they had acquired a taste for it. From this time until the 75th day they received the same limited amount of it (L) as fed to the guinea pigs. From the 75th day on the daily intake was supplemented (Sf) with 5 gm. of heated soy bean flour. Their response to the increased food was immediate. These curves show that the diet which produces scurvy in the guinea pig is adequate for the pigeon and therefore contains enough of the water-soluble vitamins to prevent polyneuritis.

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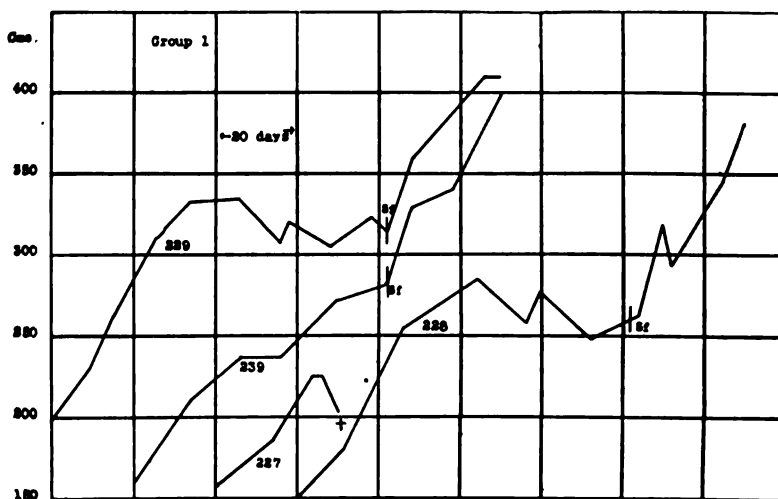


FIG. 3. Group 1. The guinea pigs in this group received daily a limited amount of the soy cake mixture and 10 gm. of raw fresh tomatoes. Animal 227 died after 30 days, showing at autopsy no macroscopic signs of scurvy. At the end of 50 days Animals 228 and 229 had apparently reached a body weight which could only be maintained and not increased by the limited food intake. To be sure that it was not the appearance of scurvy, no alterations in the diet were made until the 82nd day. At this time an addition of 5 gm. of soy bean flour (Sf) heated at 20 pounds pressure for 30 minutes was made to the diet. The response to this increased food intake was immediate. In the case of Animal 239 the heated soy flour (Sf) was added on the 62nd day and there was likewise a prompt growth response. These experiments show that the raw fresh tomato is a strong antiscorbutic agent.

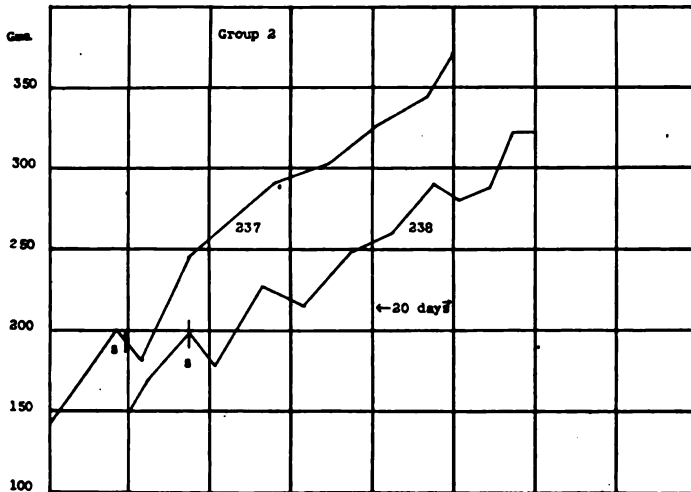


FIG. 4. Group 2. These experiments demonstrate that the raw tomato is equally effective as a curative or a preventive agent. Animal 237 on the soy cake mixture alone showed definite signs of scurvy on the 19th day. Beginning with the 20th day it received a daily supplement of 10 gm. of raw tomatoes. Animal 238 on the basal scurvy-producing diet developed positive symptoms of scurvy on the 15th day. From the 20th day on it received a daily addition of 10 gm. of raw tomatoes. Both guinea pigs responded promptly to the dietary change, becoming lively, eating better, and increasing in weight. The appearance of the first signs of scurvy, swollen and tender joints, is noted in the chart at S.

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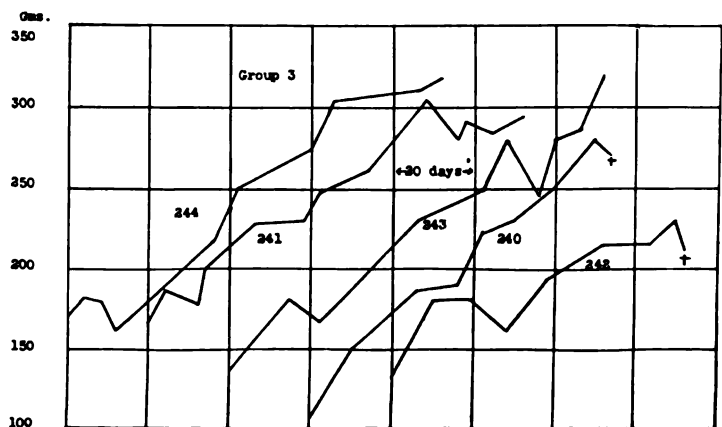


FIG. 5. Group 3. This group shows the antiscorbutic value of tomatoes dried at a low temperature (35–40°C.). A daily supplement of 1 gm. of this dried raw material uncooked has protected three of the guinea pigs during 94 days. That the limited food intake is not sufficient for their present body weight is indicated by the way two of them are just maintaining their weight. Animal 240 died on the 74th day as a result of an anatomical anomaly. It showed at autopsy no macroscopic signs of scurvy. Animal 242 died on the 72nd day from an unknown cause, showing no indication of scurvy.

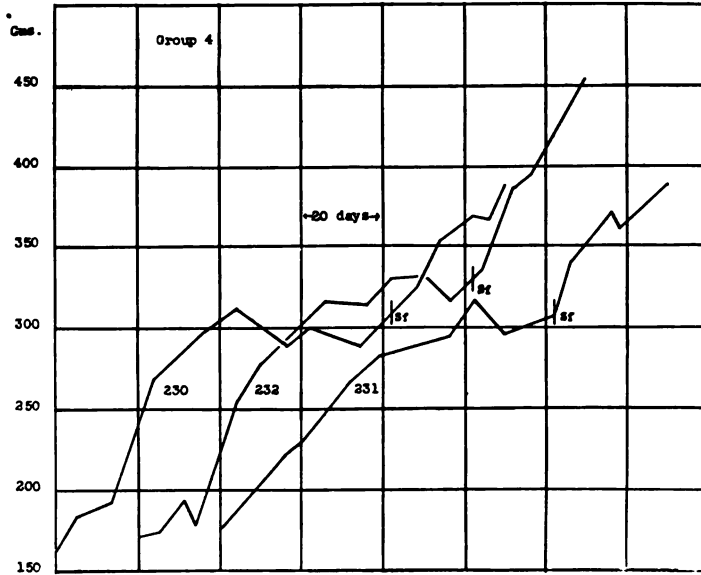


FIG. 6. Group 4. This series demonstrates that raw tomatoes dried at a comparatively high temperature still retain a significant amount of the antiscorbutic substance. A daily supplement of 1 gm. of high-dried (55–60°C.) tomatoes has protected these guinea pigs against scurvy for 110 days,—a period four to seven times as long as that in which scurvy would have developed in the absence of the supplement. It became evident after 50 days that the animals were not receiving enough food to meet their requirements. On the 82nd day 5 gm. of heated soy flour (Sf) were added daily to the food intake. The immediate response is shown by gain in weight.

THE PHYSICO-CHEMICAL STATE OF THE PROTEINS, IN COW'S MILK.*

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(From the Dairy Chemistry Laboratory, University of Missouri, Columbia.)

(Received for publication, September 13, 1918.)

INTRODUCTION.

Both qualitative and quantitative investigations regarding the proteins of cow's milk based on what may be called a mechanical separation of the several constituents date back a number of years. With the exception of Hoppe-Seyler,¹ who used an animal membrane, all the workers in this field have made use of porous clay in some form.

Herman Helmholtz had his pupil Zahn² construct a filter out of a porous porcelain cylinder, and this form of apparatus was also used by Kehrér.³ Hermann⁴ modified Zahn's method and used powdered porous plate to adsorb the casein. Tiemann,⁵ however, several years later made extensive use of a method devised by Lehmann,⁶ in which the milk was placed upon a slightly concave, porous plate, whose surface had been polished with agate. After standing for 1½ to 2 hours, the unadsorbed part, consisting of casein and fat, was scraped off and weighed. Tiemann applied this method especially to the study of the proteins of colostrum milk. Camerer and Söldner,⁷ however, have criticized Tiemann's method of analysis on the ground of the probable lack of uniformity of clay filters and also because they do not regard the casein to be completely retained on the porous plate.

* Published by permission of the Director of the Missouri Agricultural Experiment Station.

¹ Hoppe-Seyler, F., *Virchows Arch. path. Anat.*, 1859, xvii, 421-423.

² Zahn, F. W., *Arch. ges. Physiol.*, 1869, ii, 598.

³ Kehrér, F. A., *Arch. Gynäk.*, 1871, ii, 5 (cited by Schlossmann¹⁷).

⁴ Hermann, L., *Arch. ges. Physiol.*, 1881, xxvi, 442.

⁵ Tiemann, H., *Z. physiol. Chem.*, 1898, xxv, 363.

⁶ Hempel, W., *Arch. ges. Physiol.*, 1894, lvi, 558.

⁷ Camerer and Söldner, *Z. Biol.*, 1898, xxxvi, 277.

Van Slyke and Bosworth⁸ have recently made use of what they regard as a much superior form of apparatus for mechanically separating the constituents of milk, by means of which they hoped to ascertain the true state of the proteins and salts in milk. For this purpose they used a special form of Briggs'⁹ filtering apparatus, designed to obtain water extracts from soils. Van Slyke and Bosworth describe the procedure as follows:

"The process consists in putting the milk to be examined into a tubular chamber surrounding a Pasteur-Chamberland filtering tube; pressure, amounting to 40 to 45 pounds per square inch, is applied by means of a pump which forces air into the chamber containing the milk and causes the soluble portion of the milk to pass through the walls of the filter from the outside to the inside of the filtering tube, from which it runs out and is caught in a flask standing underneath."

The portion of Van Slyke and Bosworth's study of cow's milk which particularly attracted our attention and caused us to carry out the experiments presented in this paper was the conclusion that the albumin of cow's milk is partly in true solution and partly in suspension, the suspended portion being adsorbed by the casein when the milk is fresh but passing over into true solution when the milk sours or when formaldehyde is added to prevent bacterial development. These statements have been accepted as facts by certain authors.¹⁰

Our study of this question, both from a theoretical and from an experimental point of view has failed to lead to a confirmation of Van Slyke and Bosworth's conclusions regarding the condition of albumin in cow's milk. Our work did not have to do with the state of casein in milk, as the evidence which has already accumulated warrants the conclusion that it is present in fresh milk wholly in a colloidal state. Its degree of dispersion is, moreover, relatively coarse, inasmuch as cow's milk owes its familiar "milky white" appearance almost wholly to casein.

On the other hand, we feel justified in asserting that the filtration of lactalbumin through a particular Pasteur-Chamberland filtering tube, or its failure to do so is not the proper criterion by which to judge whether it is in a colloidal or true state of

⁸ Van Slyke, L. L., and Bosworth, A. W., *N. Y. Agric. Exp. Sta., Technical Bull.* 39, 1914; *J. Biol. Chem.*, 1915, xx, 136.

⁹ Briggs, L. J., *U. S. Dept. Agric., Bureau of Soils, Bull.* 19, 1902, 31.

¹⁰ Hawk, P. B., *Practical physiological chemistry*, Philadelphia, 5th edition, 1916, 313.

solution in milk. Not only does the size of the pores of the Pasteur-Chamberland tube vary,¹¹ but the size of the lactalbumin particles may be influenced greatly by the reagents used to preserve the milk. The "fresh" milk used by Van Slyke and Bosworth, from which the conclusion was drawn that the albumin is partly in colloidal and partly in true solution, was in reality milk to which 5 per cent chloroform had been added.

The data which we present in this paper show conclusively that the Pasteur-Chamberland filters which we used had a much finer porosity than those used by Van Slyke and Bosworth. We have decided to publish the data which we have already secured, since all our attempts to secure filters of any other make, which will fit the Briggs' apparatus, have been futile.

We have also made some study of another phase of Van Slyke and Bosworth's⁸ filtration studies which we believe offers a partial explanation, at least, for the data from which they conclude that "fresh" milk has its lactalbumin partly in colloidal and partly in true solution. As already pointed out, the samples of milk which they regarded as "fresh" were preserved by the addition of 5 per cent chloroform. The quantitative estimations of lactalbumin in this milk and in the serum from this milk which passed through the filter were made by the heat-coagulation method. The point which we studied was the effect of allowing both whole milk and milk free from casein to stand in the presence of 5 per cent chloroform upon this method of determining lactalbumin.

The data which we offer on this phase of the question show very clearly that chloroform left in contact with either whole milk or diluted milk serum causes a marked reduction in the amount of albumin recoverable by heat coagulation when compared with the same milk analyzed fresh. In certain cases we found this difference alone to be sufficiently great to account for the apparent result secured by Van Slyke and Bosworth that a portion of the albumin is in true and a part in colloidal solution, assuming that the filters which they used allowed the albumin particles to pass into the filtrate.

¹¹ Ostwald, W., *An introduction to theoretical and applied colloid chemistry*, New York, 1917, 47.

EXPERIMENTAL.

All of our filtration studies were made using fresh milk from the University of Missouri dairy herd. Skim milk only was used, the fat being removed by running the whole milk at least twice through a closely skimming centrifugal separator at high speed and at a temperature of 43–45°C. In no case was it possible to detect the presence of fat in this milk by the Babcock test, using a skim milk test bottle.

We found it convenient in certain of our work to make comparative studies on the same milk at the same time. This was accomplished by connecting two of the Briggs' filters to the source of pressure by a T tube. A well charged cylinder of oxygen was used as the source of pressure. With a pressure gauge in the system and good connections we were able to maintain as much pressure as the apparatus would permit.

No attempt was made to recover the heat-coagulable portion of the protein in the filtrates which passed through the Pasteur-Chamberland tubes. Whenever it was desired to separate the nitrogen in the filtrates into protein and non-protein portions, this was accomplished by the addition of 20 cc. of Almen's tannic acid reagent to 10 cc. of the filtrate, after the addition of 90 cc. of water. The precipitates which formed were allowed to stand for 24 hours before being filtered off. The nitrogen was then determined separately in the precipitate and filtrate. In a number of cases no separation of protein from non-protein was made, the total nitrogen in the serum being determined directly on suitable portions. All nitrogen determinations were made by the Gunning modification of the Kjeldahl method, using copper sulfate as catalyser.

Van Slyke and Bosworth⁸ assumed in their work that the serum passing through the Pasteur-Chamberland filters attained a constant composition after the first 50 to 75 cc. had passed through. We analyzed each of the successive 10 cc. portions of the filtrate from the first until at least 100 cc. of serum had been collected.

The Pasteur-Chamberland tubes which we used were obtained from the Central Scientific Company of Chicago.

Experiment 1. Comparison of Composition of Filtrate Using CHCl_3 and HCHO as Preservatives.—4 liters of fresh whole milk

were run through a centrifugal separator twice at 45°C., the crank being turned at a speed of 75 R.P.M. After cooling to 20°C., two portions of the skim milk of 1 liter each were carefully measured out. 50 cc. of the milk from one sample were replaced by an equal volume of chloroform, and 1.26 cc. of the other sample by an equal volume of 40 per cent formaldehyde solution. The two samples therefore contained, respectively, 5 per cent chloroform and 0.05 per cent formaldehyde. A third portion of the skim milk was subjected to immediate analysis for casein, soluble protein, and residual non-protein nitrogen, the so called soluble protein being precipitated from the casein filtrate by means of Almen's tannic acid reagent. The preserved portions of the milk were immediately placed in the filtering chambers of the Briggs' apparatus and a pressure of three to four atmospheres was applied continuously until 100 cc. of serum were obtained from the sample containing formaldehyde and 120 cc. from the sample containing chloroform. A little over 12 hours were required for the collection of the former, the latter requiring nearly 24 hours to pass through the filter.

The analysis of the fresh milk is shown in Table I, and of the successive portions of the serum from the two filters in Table II, the data representing the net results after deducting the necessary blanks for reagents, and calculating them to 100 cc. of milk.

The data presented in Tables I and II show that the Pasteur-Chamberland tubes which we used held back a great deal more of the nitrogenous constituents of the milk than those used by Van Slyke and Bosworth.⁸ It is necessary to review only a portion of the data given by these authors to show how widely divergent our results are from theirs. For example, the lowest amount of "albumin" nitrogen which they recovered from the serum amounted to 0.0232 gm. per 100 cc. of milk, and one figure reported shows as much as 0.0578 gm. of "albumin" nitrogen obtained from the serum of a sample of milk to which 5 per cent chloroform had been added as preservative. As has already been pointed out, it is clear from an experimental point of view alone that the Pasteur-Chamberland filtering tube, or for that matter any other type of porous clay filter, is not the proper criterion by which to judge whether the nitrogenous constituents

of milk other than casein are in a state of molecular or colloidal dispersion. In our trials we apparently did not even succeed in

TABLE I.

Distribution of Nitrogen in 100 Cc. of Fresh Skim Milk, Experiment 1.

Constituent.	Nitrogen.		
	Portion 1.	Portion 2.	Average.
	gm.	gm.	gm.
Casein*.....	0.3886	0.3893	0.3900
Soluble protein†.....	0.1116	0.1138	0.1127
Non-protein.....	0.0424	0.0388	0.0406

* Casein determined by official method of Association of Official Agricultural Chemists.

† Protein precipitated from casein filtrate by Almen's reagent.

TABLE II.

Comparative Distribution of Nitrogen in 100 Cc. of Serum from Pasteur-Chamberland Filter, Experiment 1.

HCHO sample.				CHCl ₃ sample.			
Sample No.	Protein N.	Non-protein N.	Total N.	Sample No.	Protein N.	Non-protein N.	Total N.
	gm.	gm.	gm.		gm.	gm.	gm.
1	—	—	0.0216	1	—	—	0.0269
2	0.0089	0.0262	0.0351	2	0.0080	0.0252	0.0336
3	—	—	0.0358	3	—	—	0.0276
4	—	—	0.0382	4	—	—	0.0269
5	0.0144	0.0268	0.0412	5	0.0007	0.0258	0.0265
6	—	—	0.0336	6	0.0033	0.0291	0.0324
7	—	0.0291	—	7†	—	—	0.0282
8	—	—	0.0375				
9	—	—	0.0398				
10	—	—	0.0415				
Average.....	0.0116	0.0270	0.0378*		0.0040	0.0269	0.0289

* Excepting Sample 1.

† Sample representing 70 to 120 cc. portion of filtrate.

recovering in the serum all the nitrogen of the milk which cannot be precipitated by tannic acid, about 35 per cent of it either

being adsorbed on the filter or existing in particles too large to pass through the pores.

Our data do, in a measure, substantiate one observation made by Van Slyke and Bosworth; namely, that more protein nitrogen passes through the filter when formaldehyde is used as a preservative than when chloroform is used. Calculation shows that only 3.5 per cent of the so called soluble proteins of the milk was recovered in the case of the chloroformed sample, while 10.3 per cent was recovered in the sample containing formaldehyde. The latter figure, however, is far short of the 92.21 per cent recovery reported by Van Slyke and Bosworth¹² for "albumin" in the case of milk containing formaldehyde. Data to be presented in a later experiment offer a probable explanation of the fact that chloroform retards the filtration of protein through the Pasteur-Chamberland filter. It will be shown there that chloroform when left in contact with lactalbumin solutions causes a partial precipitation of this protein.

Another interesting point emphasized by Van Slyke and Bosworth¹² as the result of their studies is that sour milk contains all the lactalbumin in true solution, their data showing that 100 per cent recovery of "albumin" is obtained from the serum of sour milk filtered through the Pasteur-Chamberland filter.

Experiment 2 was designed to test this point using our Pasteur-Chamberland filters.

Experiment 2. Comparison of Composition of Filtrates from Milk Containing No Preservative with Serum from Milk Whose Casein Has Been Removed by Lactic Acid.—Several liters of fresh milk were separated twice in a centrifugal separator at 45°C., the crank being turned at a speed of 75 R.P.M. 10 cc. of concentrated lactic acid were added to 1 liter of the skim milk. The casein was filtered off through cheese-cloth and the slightly cloudy filtrate filtered through a Pasteur-Chamberland tube under a pressure of three to four atmospheres. In this test the chamber of the Briggs' apparatus was surrounded by a large metal tub which was kept full of water at the temperature of

¹² Van Slyke, L. L., and Bosworth, A. W., *N. Y. Agric. Exp. Sta., Technical Bull.* 48, 1916; *J. Biol. Chem.*, 1916, xxiv, 191.

melting ice throughout the entire filtration, the outlet from the filter passing through a stopper in the bottom of the tub.

Separate analysis was made of the original milk, the nitrogen distribution being determined among the following constituents: casein, protein precipitable from the casein filtrate by Almen's tannic acid reagent, and non-protein nitrogen.

The filtration was relatively rapid in this test. Analyses were accordingly made of the first ten 10 cc. portions and of the next three 50 cc. portions, the last analysis thus representing the portion from 200 to 250 cc.

For comparison with the data secured in this test we offer the data secured from the filtration of a sample of skim milk, prepared at another time, the principal point of comparison being that it, too, was filtered at the temperature of melting ice, without the use of any preservative. No nitrogen distributions were made in this case, however, total nitrogen determinations only being made upon the first ten 10 cc. portions which passed through the filter. The casein and total non-casein nitrogen were determined in a separate portion of the milk. The data from this experiment are shown in Tables III and IV, Table III giving the analyses of the original samples of milk and Table IV the composition of the various portions of the serum.

In spite of the fact that the two samples of milk represented in this experiment were of entirely different composition and their filtration was carried out at different times, and also notwithstanding the fact that Sample II was merely the lactic acid whey, free from casein, there is a marked uniformity in the proportion of the non-casein nitrogen which passed through the filter in the case of the two samples. It is evident also that in both cases this consisted almost wholly of non-protein nitrogen and shows that our filters had a porosity of such fineness as to retain most of the proteins other than casein even after they had been rendered "soluble," as Van Slyke and Bosworth believe, by the lactic acid, and there was no casein present to adsorb them. We interpret this to mean that the albumin of sour milk is still in a colloidal condition and that it is not to be regarded as in true solution simply because a porcelain filter whose pores are of a certain degree of fineness will allow it to pass.

Experiment 3. Effect of Chloroform on Determination of Lactalbumin by Heat Coagulation.—The method most commonly used

TABLE III.

Distribution of Nitrogen in 100 Cc. of Original Milk, Experiment 2.

Constituent.	Sample No.	Nitrogen.		
		Portion 1.	Portion 2.	Average.
		gm.	gm.	gm.
Casein N.....	I*	0.4308	0.4321	0.4315
Non-casein N.....	I*	0.1557	0.1505	0.1531
Casein N.....	II†	0.4500	0.4494	0.4497
Protein N other than casein.....	II†	0.1007	0.1007	0.1007
Non-protein N.....	II†	0.0255	0.0268	0.0262
Total non-casein N.....	II†	—	—	0.1269

* Fresh skim milk filtered without any preservative at temperature of melting ice.

† Fresh skim milk, the lactic acid whey from which was filtered without preservative at temperature of melting ice.

TABLE IV.

Relative Distribution of Nitrogen in 100 Cc. of Serum from Pasteur-Chamberland Filters, Experiment 2.

Fresh milk.		Lactic acid whey.			
Sample No.	Total serum N.	Sample No.	Protein N.	Non-protein N.	Total N.
	gm.		gm.	gm.	gm.
1	0.0332	5	0.0038	0.0249	0.0287
2	0.0527	6	—	—	0.0323
3	0.0513	7	—	—	0.0336
4	0.0533	8	—	—	0.0352
6	0.0414	9	—	—	0.0340
7	0.0440	10	0.0064	0.0271	0.0335
9	0.0427	12	0.0064	0.0289	0.0353
10	0.0447	13	0.0064	0.0262	0.0326
Average*.....	0.0471	Average.	0.0056	0.0268	0.0331
Percentage of same nitrogen in original milk.....	30.7		5.5	100.0	26.0

* Excepting Sample 1.

for the quantitative estimation of lactalbumin in cow's milk is based on a coagulation of the casein filtrate by heat. The protein recovered by this method does not, however, represent all of the protein present in the casein filtrate. In every case, even after the most careful coagulation, it is possible to recover considerable protein from the "albumin" filtrate by tannic acid, and, in the senior author's experience this fraction is frequently greater than that thrown down by heat coagulation. Even this procedure leaves a small quantity of nitrogen which is evidently not of true protein nature, since it fails to respond to the most delicate tests for protein.

It has long been a disputed point whether the non-coagulable protein of milk serum is to be regarded as of a proteose-peptone character. It is not our purpose to review this controversy at this time, but it may be pointed out that there never has been any conclusive proof offered that fresh milk contains these secondary proteins. It is the author's opinion that much of this controversy has arisen because of the failure to appreciate the fact that heat coagulation seldom if ever gives a quantitative measure of the so called soluble animal proteins. This is particularly applicable to the albumin and globulin in milk. This has not been properly appreciated by dairy chemists in recent years, notwithstanding the fact that foreign investigators have been emphasizing it for nearly 50 years.

Guth¹² as long ago as 1870, pointed out that heat does not give a complete precipitation of albumin from milk. Hofmeister,¹³ applying this method to animal fluids in general, found that it rarely leaves the filtrate free from protein. Neumeister,¹⁴ studying the question of the presence of pyrenin in milk, concluded that heat coagulation in weakly acid solutions always results in the formation of primary proteoses from the coagulated pyrenin, which he regards as especially easily hydrolyzed. Sebelien¹⁵ found in his extensive investigation of milk proteins that a large part of the albumin of milk remained uncoagulated at the boiling temperature, as high as one-fourth to one-third of the total albumin being lost. Schloss-

¹² Guth, *J. W. Chem. Soc. Trans.* 1870, iii, 78.

¹³ Hofmeister, *J. Z. Naturf. Chem.* 1878-79, ii, 288.

¹⁴ Neumeister, *N. Z. J. Biol.* 1888, viii, 272.

¹⁵ Sebelien, *J. Z. Naturf. Chem.* 1889, xiii, 155.

mann,¹⁷ reviewing the various methods of estimating milk proteins, also points out that it is impossible to precipitate completely the albumin by heat.

These statements, which are by no means claimed to be a complete bibliography on the subject, only serve to confirm the author's own conviction based upon a wide experience and much study of the question of the quantitative determination of the lactalbumin in cow's milk. The writer hopes to publish some of his own experimental work on this question in the near future.

TABLE V.

Distribution of Protein in 100 Gm. of Milk when Fresh and after Standing 7 Days with 5 Per Cent CHCl₃.

Sample No.	Constituent.	Fresh skim milk.	Skim milk after 7 days with CHCl ₃ .	Variation from original.	Casein filtrate after 7 days with CHCl ₃ .	Variation from original.
		gm.	gm.	per cent	gm.	per cent
I	Heat-coagulable protein.....	0.315	0.239	-40.0	—	—
I	Residual protein.....	0.322	0.328	+1.7	—	—
II	Casein.....	2.932	2.889	-1.4	—	—
II	Heat-coagulable protein.....	0.405	0.370	-8.6	—	—
III	Casein.....	3.016	2.800	-7.1	—	—
III	Heat-coagulable protein.....	0.442	0.299	-32.4	0.074*	-13.8
					0.297	
III	Residual protein.....	0.301	0.417	+38.5	0.384	+27.6
III	Non-protein nitrogen.....	0.030	0.055	+83.3	0.044	+46.7

* Precipitate which formed on standing.

In their studies on the filtration of "fresh" milk through the Pasteur-Chamberland filter, Van Slyke and Bosworth⁸ determined the albumin in both whole milk and milk serum by coagulation with heat. Not only does this method leave a large part of the albumin in the filtrate, but the amount of albumin which can be recovered by this method is likely to be still less because of the presence of chloroform in the milk, which was the preservative used for their "fresh" milk studies.

The data presented in Table V show the results of several experiments in which the heat-coagulable proteins were determined

¹⁷ Schlossmann, A., *Z. physiol. Chem.*, 1896-97, xxii, 197.

in samples of fresh, unpreserved milk, and again in the same milk after standing for several days in the presence of 5 per cent chloroform. In the case of Sample III, additional information on this point was secured by precipitating the casein from a separate portion, making the filtrate and washings up to a volume of 200 cc., adding 10 cc. of chloroform, and completing the analysis after standing for several days at room temperature. The method used for coagulation of the albumin was to neutralize the casein filtrate with NaOH solution, using phenolphthalein as indicator, add 0.3 cc. of 10 per cent acetic acid solution, bring the solution rapidly to the boiling point, and boil gently for 10 minutes. The coagulum was then filtered off and washed, and the nitrogen determined, as in all other cases, by the Kjeldahl-Gunning method. The data presented are the average of duplicate determinations.

Columns 5 and 7, Table V, show the percentage variation of the second determinations from the original analyses. The point which it is desired to emphasize is the loss in heat-coagulable protein in each case, ranging from nearly 10 per cent in Sample II to 40 per cent in Sample I. An interesting point which developed in Sample III in connection with the casein filtrates which were allowed to stand in contact with chloroform was the gradual formation of a white precipitate. This was filtered off and its nitrogen determined separately. The duplicate determinations of this precipitate were, respectively, 0.080 and 0.068 gm. of protein. When considered with the protein recovered by heat coagulation, the loss from the original analysis was 13.8 per cent, but the portion recovered by heat alone was practically identical with that recovered from the skim milk in contact with the chloroform.

The data seem to be very suggestive of the probable cause of the major part of the loss in heat-coagulable protein when milk is preserved with chloroform. They also offer a very plausible explanation of the apparent result secured by Van Slyke and Bosworth⁸ that chloroformed milk allows much less heat-coagulable protein to filter through the Pasteur-Chamberland filters than milk containing formaldehyde. We have so far been unable to find any theoretical grounds to support the idea that chloroformed

milk represents more nearly the natural condition, so far as the state of the protein is concerned, than milk to which formaldehyde had been added in amount sufficient merely to prevent bacterial development. Our own experience is also contrary to this idea.

The authors do not consider it likely that proteolysis was a factor in causing the results indicated in Table V.

SUMMARY AND CONCLUSIONS.

Experiments are reported in which fresh skim milk, skim milk preserved with 5 per cent chloroform, skim milk preserved with 0.05 per cent formaldehyde, and the lactic acid whey from fresh skim milk were filtered through Pasteur-Chamberland filtering tubes under pressure. The total protein passing through the filters was determined in each case by precipitation with Almen's tannic acid reagent, and the non-protein nitrogen in the filtrate from the precipitate thus formed.

The amount of non-casein protein recovered in the filtrate did not in any case exceed 10 per cent of the non-casein protein in the original milk, and in most cases the amount recovered was considerably less than this figure. There was also only a partial recovery of the non-protein nitrogen of the original milk in the experiments with chloroformed and formaldehyde-treated milk. These results are widely divergent from those reported by previous investigators.

The variation in the size of the pores of different Pasteur-Chamberland filters which is indicated by a comparison of our data with those secured by others who have filtered milk through these filters shows conclusively the fallacy of drawing conclusions regarding the true state of solution of non-casein proteins of milk based on filtration studies of this character.

Some remarks are offered regarding the fallible practice of determining the "albumin" of milk by heat coagulation. Several experiments are reported showing how chloroform left in contact with milk greatly depresses the amount of protein which can be recovered from casein filtrates by this method. One experiment is reported indicating that this depression may be due partially

to a precipitation of heat-coagulable protein by the chloroform. A plausible explanation is thereby offered for the results secured by other investigators who find that chloroformed milk apparently allows less heat-coagulable protein to pass through the Pasteur-Chamberland filter than milk preserved with formaldehyde, or sour milk.

1st Lieutenant Robert G. Scott, aerial observer, was killed in action on October 4, 1918.

Endowed with a brilliant mind, a splendid physique, and a tireless energy, his keen interest in biochemistry gave rich promise of a splendid future. Courageous and dauntless to a fault, his country's call naturally led him into the field of service which he chose. Possessed with a happy disposition, he greatly endeared himself to his associates who mourn his loss to science at the threshold of his career.

THE STATE OF PROTEINS IN COW'S MILK.

By L. L. VAN SLYKE AND A. W. BOSWORTH.

(Received for publication, October 21, 1918.)

In the preceding article Palmer and Scott have drawn certain conclusions concerning the condition of albumin in cow's milk, based upon the separation of serum from milk by a method outlined by us in a previous paper.¹ This method involves filtration through porous clay filters. In our paper we stated:

"It has been found by Rupp that the filter appears to have the power of adsorbing some of the constituents of the serum until a volume of 50 to 75 cc. has passed through, after which the filtered serum is constant in composition. In our work, therefore, the first portion of serum filtered is not used."

We did not "assume that the serum passing through the Pasteur-Chamberland filter attained a constant composition after the first 50 to 75 cc. had passed through," but actually found this to be the case, giving credit to Rupp for the original observation, however. That this is the case is also shown by the figures given by Palmer and Scott in their Table II. In the case of the milk treated with formaldehyde the total nitrogen in the serum of the first ten 10 cc. portions varied from 0.0216 to 0.0415 gm. As they used much finer filters than we did it would require the passage through them of a greater volume of liquid in order that they might become saturated with respect to those substances adsorbed. Had they discarded the first 100 cc. or so and then collected a total of 250 cc. or more from which to draw samples for analysis the results obtained by them would have more weight when used to criticize the results secured by us.

With respect to their work on chloroformed milk we can only say that the action of chloroform upon the proteins of milk is a

¹ Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 135.

progressive one, due to two or more factors, one of which is the action of the hydrochloric acid produced. After a few weeks the proteins may be completely precipitated from the milk. The changes noted by them at the end of 7 days are therefore not comparable to the changes which might have taken place in our filtrations lasting from 12 to 36 hours.

THE DIETARY PROPERTIES OF THE PEA (VICIA SATIVA).*†

By E. V. McCOLLUM, N. SIMMONDS, AND H. T. PARSONS.

(From the Laboratory of Chemical Hygiene, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.)

(Received for publication, December 19, 1918.)

The pea and the navy bean have become better established as staple human foodstuffs in the United States than have any other of the legume seeds, but in recent times the cow-pea, soy bean, and peanut have been promoted. The soy bean is second only to rice in importance as a foodstuff in the Orient. Several studies have been made of the nutritive values of certain of the isolated proteins from these seeds by Osborne and Mendel (1), and to some extent the peanut and soy bean have been investigated in their dietary properties by Osborne and Mendel (2), Daniels and Nichols (2), and Daniels and Loughlin (2). These authors have attributed unusually high values to both of these seeds, especially with respect to the quality of their proteins and the content of fat-soluble A. From their data one would conclude that they are dietetically unique among seeds in these respects.

For several years we have been engaged in the study of the special dietary properties of our more important foodstuffs, with a view to discovering the exact nature of their deficiencies from the dietary standpoint. These studies have now included all the more important cereal grains (3), wheat germ (4), the navy bean (5), certain leaves (6), mixtures of cereal grains and the legume seeds (7), and complex mixtures of seeds including both the cereal

* A part of the experimental work reported in this paper was carried out by the authors at the Wisconsin Experiment Station.

† We employed split peas which were soaked in distilled water and heated 75 minutes in an autoclave at 15 pounds pressure; they were then dried in a current of air about 70°C., and ground.

grains and the legume seeds, especially the pea and bean (8). The results of these studies have led us to formulate a new classification of the foodstuffs based on their biological functions (9). Among the vegetable foods, those which are functionally storage tissues as the seeds, tubers, certain roots, and to some extent certain modified leaves, as the thick leaf of the cabbage, show decidedly greater dietary deficiencies than do those which are the seat of great metabolic activity. Examples of the latter class are the leaves generally, but more especially the thin leaves which are not filled with reserve food materials, the germ of the seed, and probably also those regions of the tubers and fleshy roots which are rich in cellular elements. The seed, tuber, and root group are all decidedly deficient in calcium, sodium, and chlorine among the inorganic elements, and in the quality of their proteins, and with few exceptions in the content of fat-soluble A. The leafy portions of the plants approximate complete foods, and in some plants are actually complete foods which suffice for years to maintain a state of good nutrition in animals. An example in point is the prairie grass on which the bison subsisted throughout the year. It is true they had access to salt licks and manifested a distinct craving for salt. There seems on the other hand to be no mammal which is able to subsist entirely upon a diet of seeds, but certain birds when fed a supplementary supply of calcium in water, shells, grits, etc., appear to be satisfactorily nourished on such a restricted diet. It is interesting to note that millet seed, which among the seeds is especially rich in fat-soluble A, has found favor with bird fanciers as a regular constituent of rations.

The present paper contains a report of the dietary properties of the common pea. The data are recorded because of their interest in contributing to the evidence in support of the general theory which we have formulated; *viz.*, that vegetable foods having similar functions have likewise similar dietary properties (10).

We also present records of experiments which show the supplementary relationships between the proteins of the pea and casein, gelatin, zein, and lactalbumin, respectively. It is of great interest that while both casein and zein supplement the deficiencies of the pea proteins, gelatin and lactalbumin do not. From the failure of lactalbumin to supplement the proteins of the pea, or to induce growth when fed in the amounts used in the experi-

ments described in this paper, we have come to the tentative conclusion that lactalbumin is either an incomplete protein or a poorly constituted one. If lactalbumin were actually so good a protein as the experiments of Osborne and Mendel indicate, the 9 per cent with which we supplemented a content of 10 per cent of pea protein in Lot 767, Chart 5, should itself have been sufficient to induce good growth. We have elsewhere shown that 8 per cent of protein derived from the mixture of proteins contained in milk, is sufficient to lead to nearly normal growth (11), and 9 per cent of a protein mixture, two-thirds of which is derived from rye and the remainder from flaxseed oil meal, is of such good quality that with this amount in the diet young rats grow at the optimum rate to full adult size (12).

We have been unable to obtain any growth in young rats with diets containing 18 per cent of lactalbumin as the sole source of protein (8), when the remainder of the food mixture was so constituted that good growth could be secured when the protein (18 per cent) of the diet was casein. We are forced to the conclusion that lactalbumin is a poorly constituted or an incomplete protein, and that the excellent results of Osborne and Mendel were due to the high proportion of nitrogen derived from "protein-free milk" which was present in their food mixtures and served to supplement the lactalbumin with respect to some as yet undetermined cleavage product which is essential for growth (13).

Zein is lacking in glycocoll and tryptophane and contains little, if any lysine, and is very low in cystine, if indeed this amino-acid is present. Since the proteins of the pea are fairly well supplemented by zein, it follows that the limiting amino-acid in the pea is neither tryptophane, lysine, nor cystine (see Chart 8). Glycocoll has been shown by McCollum and Hoagland (14) to be readily synthesized by the mammalian organism.

Chart 1.—Lot 866 illustrates the slow rate of growth with early stunting, of young rats which were fed peas sufficient to make about 20 per cent of protein, supplemented with a salt mixture¹ and fat-soluble A (in butter fat). A comparison with Lots 976, 903, and 1030 (Charts 1 and 2) shows that the peas were supple-

¹ For composition of salt mixture 185 see McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxii, 191.

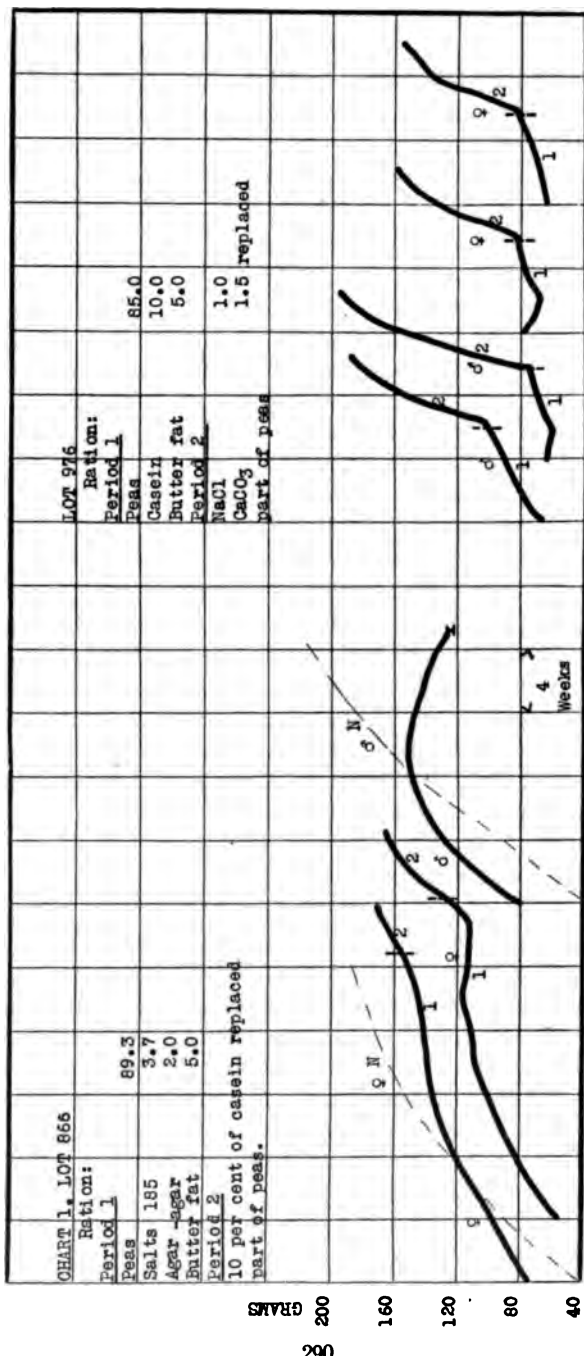


CHART 1.

mented with respect to all necessary factors except protein. In Period 2, the addition of casein led to a sharp response with growth in one rat, and a distinct acceleration of growth in another. This shows that the pea proteins are of very poor quality when fed as the sole source of nitrogen. The rate of growth on this diet containing 20 per cent of pea protein is not so good as has been observed with diets containing but half as much protein derived from one of the cereal grains (3). That the addition of a salt mixture is necessary is shown by the records of Lot 976, Chart 1. The pea is fairly rich in the fat-soluble A as is shown by Chart 2, Lot 1030.

Lot 976, Period 1, shows that even when supplemented with protein and fat-soluble A, peas to the extent of 85 per cent of the food mixture cannot support growth, because of the shortage of certain inorganic elements. Period 2, shows that no inorganic additions other than calcium, sodium, and chlorine are necessary in order to make the diet satisfactory for the support of growth at the optimum rate. We have not, up to the present time, found any seed or mixture of seeds which did not require inorganic salt additions before growth could take place.

Chart 2.—Lot 903, Period 1, illustrates as did Chart 1, Lot 866, that the proteins of the pea are not of very good quality for the support of growth. 45 per cent of peas furnishes a protein content of about 10 per cent of the food mixture, but the animals were not able to grow on this diet even though the peas were supplemented with respect to all other factors. This amount of protein from one of the cereal grains would suffice for the support of growth at a better rate (3). In Period 2, when 9 per cent of casein was added, there was a prompt response with growth in all animals. Peas are not so high in hemicelluloses as are navy beans, and fermentation of indigestible carbohydrates is not so depressing a factor as in diets containing a high content of navy beans. If the proteins of the pea were of good quality it should be apparent from the behavior of animals fed the types of diets which we have employed.

Lot 1030 illustrates the remarkable growth and good reproduction records of rats which were restricted from weaning to a diet of peas supplemented with casein, calcium carbonate, and sodium chloride. One female had four litters of young, but none

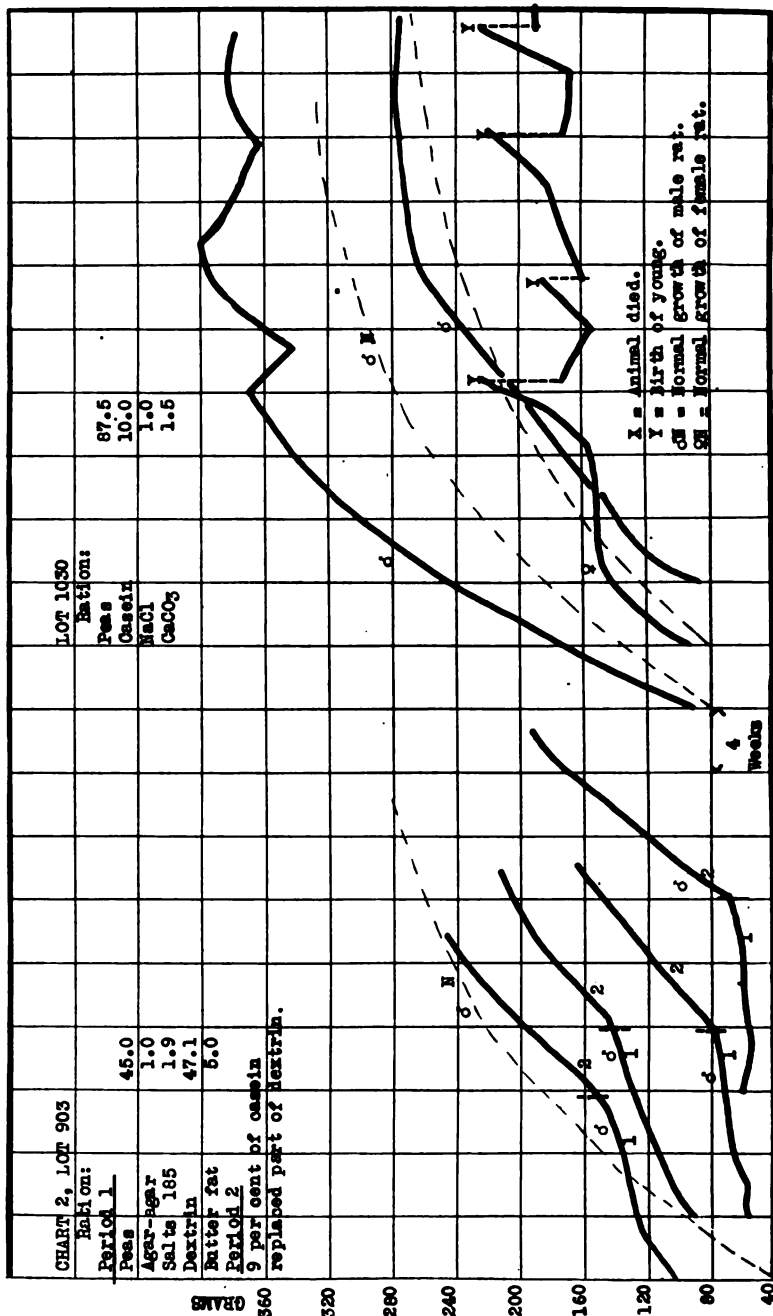


CHART 2.

of them were reared. The other at the age of about 10 months had her first and only litter of young, but these died when 15 days old. This diet would have been improved by the addition of butter fat (fat-soluble A) (see Chart 7, Lot 757). It is interesting to observe that without addition of the latter factor, such good growth and reproductive capacity could be secured. The results of this experiment demonstrate that there can be no very serious injury resulting from the feeding of a liberal amount of peas over a long period of time. In this respect peas appear to be superior to the navy bean which seems to cause injury when fed in liberal amounts to growing rats (5). Lathyrism has, so far as we are aware, not been observed with the species of pea we have employed in our experiments, but only with *Lathyrus sativus* (15) which belongs to the sweet pea family.

Chart 3.—Lot 754 illustrates well the great importance of adequately supplementing a diet in which one seed furnishes the major portion of the diet, with salts to supply suitable inorganic additions and with protein. This food mixture was similar to that of Lot 1030, Chart 2, except that it contained butter fat, and a complex salt mixture, instead of a simple addition of calcium carbonate and sodium chloride. This was one of our earlier experiments and the salt mixture was such as to furnish qualitatively all the elements except iodine which are needed by a growing animal, but the elements calcium, sodium, and chlorine were not furnished in such liberal amounts as in Lot 1030 or as to promote optimum nutrition. This was apparently the cause of the failure of Lot 754 to grow as well as the former.

We wish to emphasize that peas supplemented with respect to protein, fat-soluble A, and with a complex salt mixture, as described in Lot 754, Chart 3, failed to be satisfactorily nourished. Lot 757, Chart 7, which contained but half as much peas and was supplemented with essentially the same purified additions, was much better nourished as shown by both growth curves and reproduction records. In the latter group the number of young produced may be regarded as normal since two females produced nine litters (thirty-eight young), but none were successfully weaned. Such results strongly suggest the presence in peas of some substance or substances which prove injurious when taken in large amounts. It seems, however, that the toxicity of peas, if there

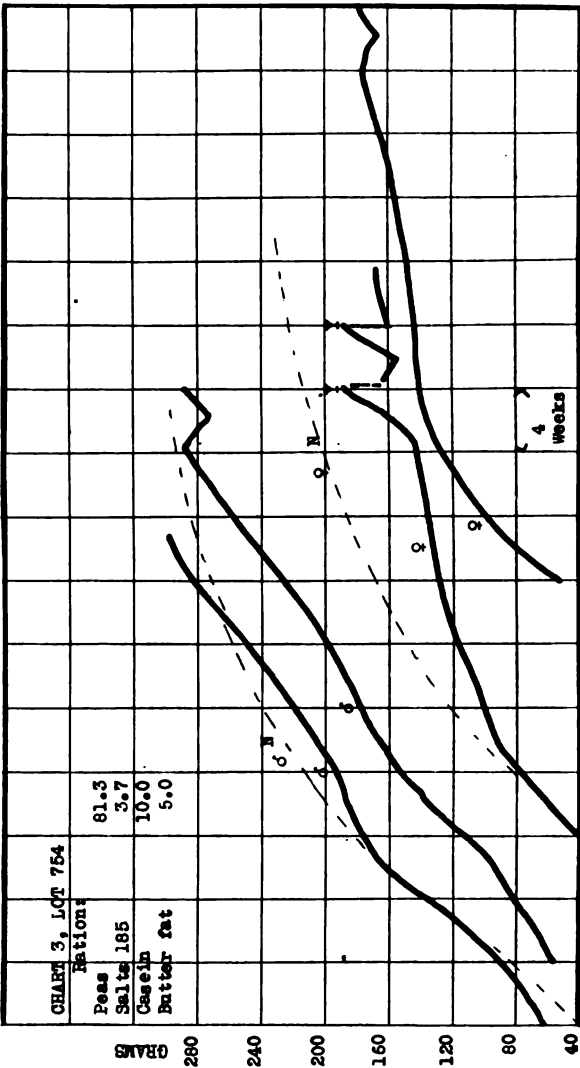


CHART 3.

be any, is but slight and only manifests itself when diets extremely rich in peas are persisted in over a long period. In the quantities in which peas ever enter into human dietaries there can be no danger of the development of such a syndrome as lathyrism, but there may be a depression of function when the diet is distinctly unsatisfactory in some other factor.

Chart 4.—Lot 627 was fed a diet consisting of a purified food mixture supplemented with butter fat (fat-soluble A) and 5 per cent of peas. The latter was the sole source of the dietary factor water-soluble B. The animals were able to grow for a month, after which they failed rapidly. It is evident that this amount of peas is entirely insufficient to furnish the antineuritic factor, for two rats suffered from polyneuritis after 8 and 10 weeks respectively. Even so small an amount of wheat germ as 2 per cent of the food mixture suffices for the support of growth at nearly the normal rate when the diet is otherwise composed of purified foodstuffs and butter fat to furnish fat-soluble A (16). The cell-rich tissues of the plant such as the germ are better sources of the water-soluble B than are the entire seeds, but these in turn are better than is the endosperm.

Lot 695 was fed a diet which contained 25 per cent of cooked, dried peas, and consisted otherwise of purified foodstuffs and butter fat to furnish the fat-soluble A. Although the peas had been cooked under pressure of 15 pounds for $1\frac{1}{2}$ hours and dried in a current of hot air, one-fourth of the food mixture derived from peas so treated sufficed to supply enough of the water-soluble B to enable young rats to grow to the full adult size at the normal rate. Further studies are in progress to determine the minimum amounts of each of the more important seeds which meet the requirements of the growing young for this dietary factor. For the pea, it lies somewhere between 5 and 25 per cent of the food mixture (8). The amount of water-soluble B which is sufficient for growth is not necessarily enough for successful rearing of young. The demands of the nursing mother for this factor appear to be much greater than for the young for growth after the weaning period (17).

Chart 5.—Lot 767 presents curves of growth which are of remarkable interest in that they show that the proteins of the pea are not supplemented by lactalbumin. This protein has been

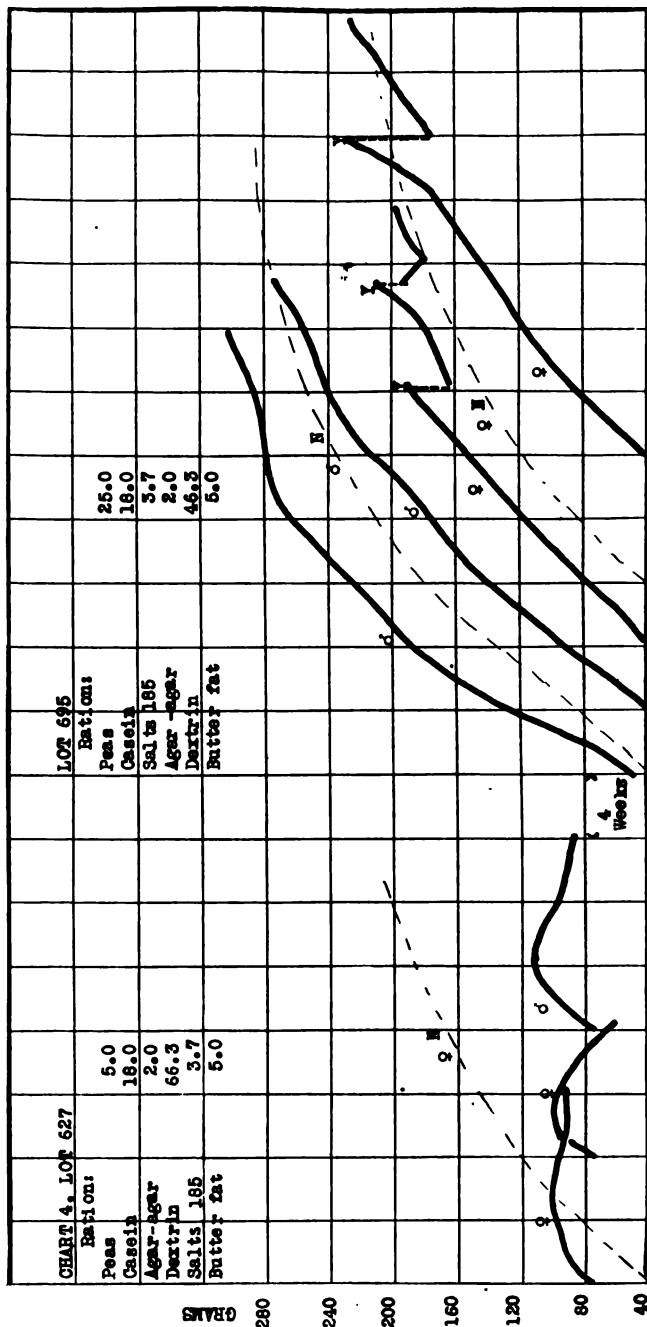


CHART 4.

said by Osborne and Mendel to be of greater biological value than any of the other purified proteins studied (13). If this is true we must accept the view that it is highly satisfactorily constituted with respect to its content of all the essential amino-acids and can be converted into tissue proteins to an extent nearer quantitative than can any other protein thus far studied. If this were true, 9 per cent of lactalbumin should be capable of supporting growth at a rate approximately as great as is possible in this species.

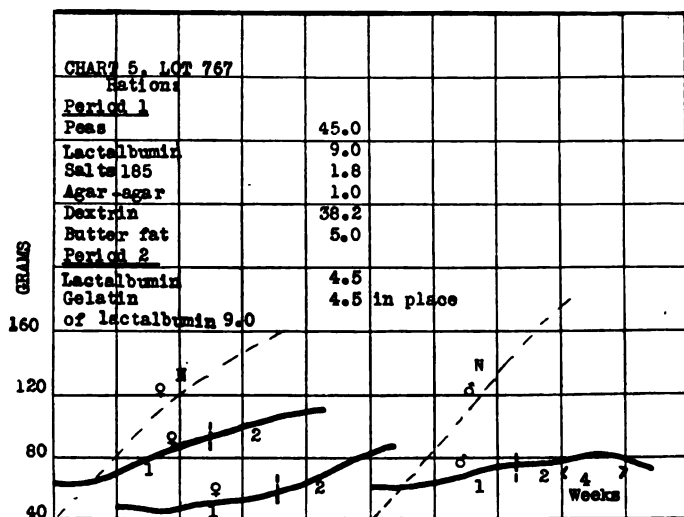


CHART 5.

Chart 6.—Lot 756 shows gelatin, like lactalbumin, to be unsuited for making good the deficiencies of the pea proteins. In Period 1 the diet was similar in all respects to that of Lot 757, Chart 7, except that gelatin replaced casein, in like amount. The diet containing the casein together with pea protein induced growth, whereas that containing gelatin and pea proteins did not. In Period 2, the diet was made up with 9 per cent of casein replacing the gelatin. There was an immediate response with growth in all the animals.

Chart 7.—Lot 757. In marked contrast to lactalbumin and gelatin, casein is a good protein for supplementing the deficiencies of the proteins of the pea. The diet of this group was similar in

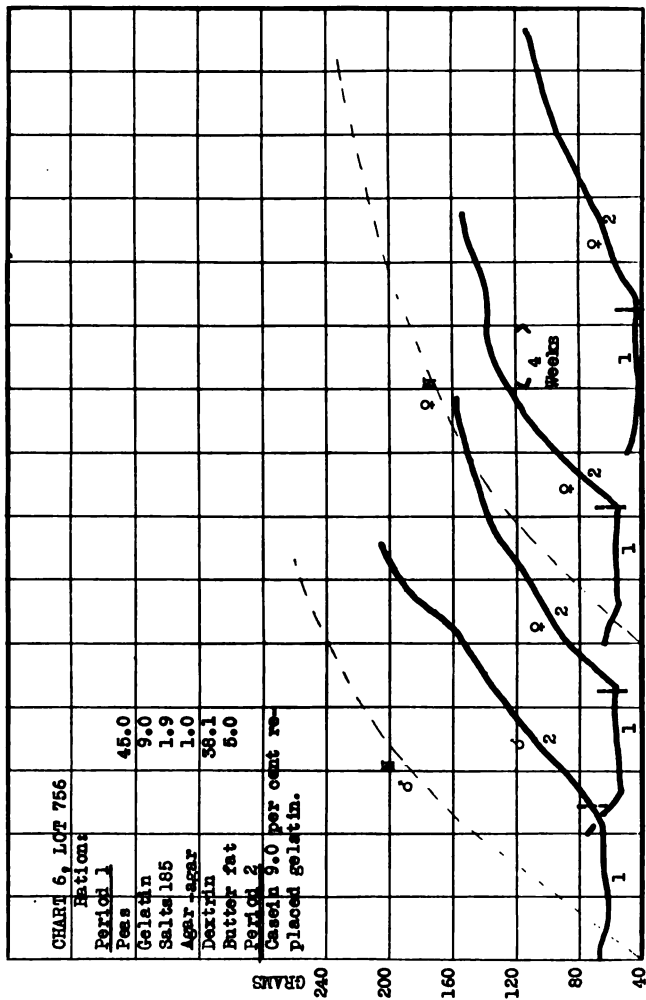


CHART 6.

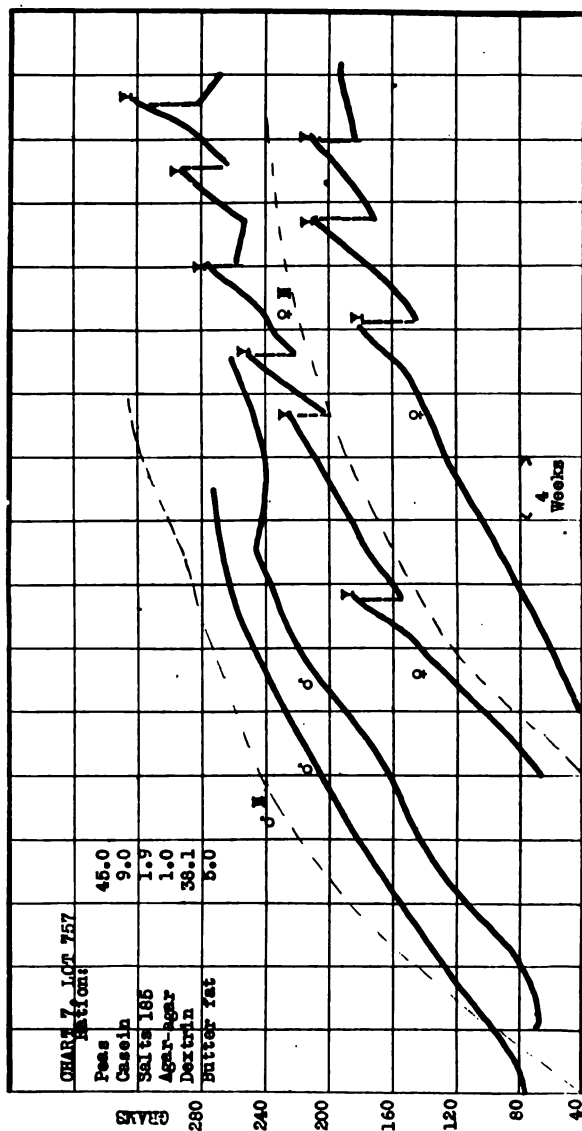


CHART 7.

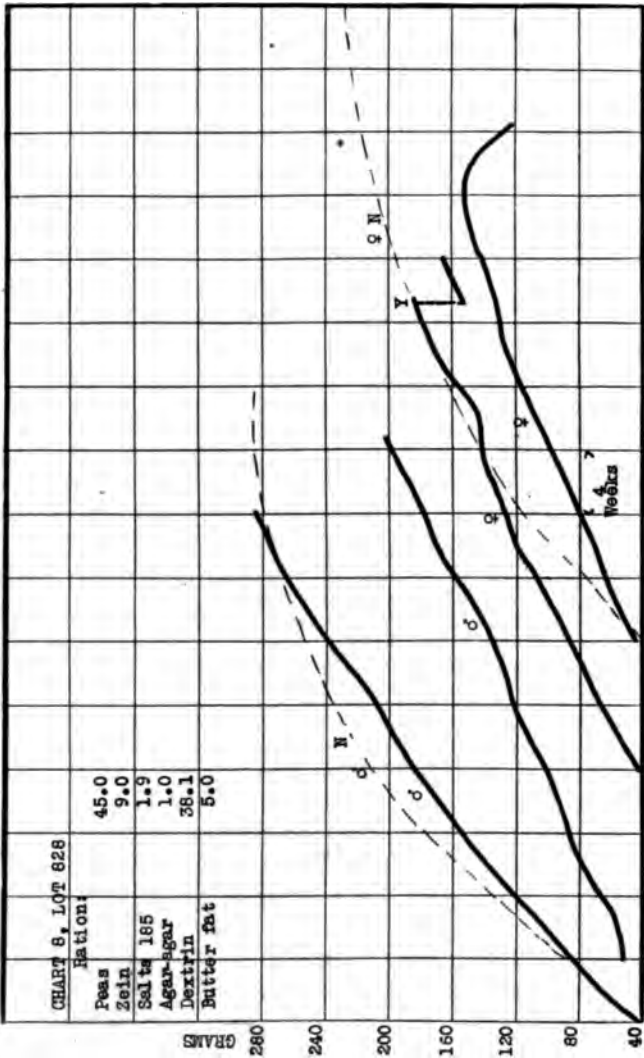


CHART 8.

every respect to that of Lot 767, Chart 5, except that in the latter lactalbumin replaced casein. This supports the view that lactalbumin is a poorly constituted protein.

Chart 8.—Lot 828 makes it clear that zein is a moderately good supplementary protein for the proteins of the pea. The value of the mixture of approximately equal parts of zein and pea proteins is apparently distinctly greater than half that of an equal amount of casein, for about 15 per cent of the latter is required when the diet is otherwise satisfactory to promote growth at about the normal rate (18).

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MODIFICATIONS OF BENEDICT'S AND FOLIN'S QUANTITATIVE SUGAR METHODS.

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Benedict's method¹ has proved satisfactory for estimation of sugar in urine when the concentration is above 0.2 per cent. Folin's² modification of Benedict's method is especially useful for micro-titration of urine containing much sugar. Since both these methods require potassium thiocyanate, which unfortunately has been unobtainable for some time, the writer substituted for it sodium thiocyanate which is easily secured.

Benedict's solution was prepared using 105 gm. of NaCNS (equivalent to 125 gm. of KCNS) and proved to be a perfect substitute for the usual reagent. The titrations with urines were identical with the regular reagent and with the substitute. In preparing the reagent we dissolve the sodium citrate first in 650 cc. of hot water, then the (monohydrated) sodium carbonate, and finally the sodium thiocyanate. After filtering the solution through cloth, the copper sulfate solution is added without waiting for it to cool.

On substituting NaCNS for KCNS in Folin's mixture of salts titration figures were obtained that were distinctly too low. The same result was secured when the thiocyanates were weighed out separately (from the phosphate and carbonate) for each estimation, thus eliminating the possibility of the error being due to a lack of uniformity in the mixing of the salts.

After experimenting it was found that correct titrations could be obtained by reducing the amount of sodium thiocyanate somewhat, and using periods of boiling half as long as those di-

¹ Benedict, S. R., *J. Am. Med. Assn.*, 1911, lvii, 1193.

² Folin, O., and McEllroy, W. S., *J. Biol. Chem.*, 1918, xxxiii, 513.

rected by Folin (0.5, 1.0, and 1.5 minutes instead of 1, 2, and 3). The results were as close to those secured by Folin's technique as duplicate estimations by the latter method; in other words, practically identical. The comparison was made with several different concentrations of sugar (urines) between 0.5 and 5.5 per cent. Both with slow titration (six to eight portions of urine added) and with rapid titration (three portions) the results were satisfactory.

For each estimation we use 4 gm. of a salt mixture containing 20 gm. of sodium thiocyanate, 60 gm. of dry sodium carbonate, and 100 gm. of disodium phosphate. We have found it convenient to use a small flask for the analysis instead of a test-tube.

THE INCREASE IN NITROGEN METABOLISM OF THE DOG, FOLLOWING THE ADMINISTRATION OF DESICCATED THYROID GLAND.

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(Received for publication, January 2, 1918.)

INTRODUCTION.

The following experiments were undertaken to determine how serviceable are small doses of commercial desiccated thyroid gland administered by mouth, as a means of increasing nitrogen elimination in the dog. That thyroid gland preparations cause an increase in nitrogen elimination in dogs has been reported in carefully balanced feeding experiments (1-10). The application of these findings to experimental work is so frequent as to justify additional study of this subject in the hope of making the procedure more practicable. We have undertaken to follow the nitrogen excretion on dogs which received only sugar solutions, thus eliminating the determination of a nitrogen intake, and to obtain figures for a dosage which would produce a marked increase in the nitrogen elimination.

EXPERIMENTAL.

The following general procedure was carried out for each experiment. A healthy, well nourished animal was kept for 24 to 48 hours on water, then each day within $\frac{1}{2}$ to 1 hour of the same time the bladder urine was removed by catheter, the bladder washed free of urine, the animal weighed, and a measured amount of sugar water given by stomach tube. The sugar solutions contained a mixture of 125 gm. of cane sugar and 62 gm. of glucose to 1,000 cc. of water. Thyroid or dried veal pills when administered were placed in the throat before feeding the sugar water by stomach tube. A mixture of equal parts of desiccated thyroid preparation from Armour and Company, and from Parke, Davis and Company was used. This preparation contained 10 per cent

TABLE I
Summary of Literature on Thyroid Preparations Administered to the Dog by Mouth.

Experimenter.	Experiment No.	Weight of dog.	Substance.	Dose.	Food.	Average daily N balance.	N balance day of administration.	Average increase in N in urine.	Increase in N on day of administration.	Days of administration.	Remarks.
Roos (1)	1	8.5	Dried and powdered thyroid.	3 (1 dose)	Dog biscuit, milk.						
	2	7.9	" " "	6 (1 ")							
	3	12.1	" " "	5 (1 ")							
	4	11.9	Dried thyroid.	5 (1 ")							
	5	12.1	" " "	5 (1 ")							
	6	12.6	" " "	5 (1 ")							
	7	13.0	" " "	5 (1 ")							
	8	13.0	" " "	5 (1 ")							
	9	13.7	" " "	5 (1 ")							
	10	13.9	" " "	5 (1 ")							
	11	14.4	" " "	5 (1 ")							
	12	10.2	Thyroidine.	0.05 (1 ")							
	13	10.1	" " "	0.06 (1 ")							
Roos (3)	14	11.1	Preparation from human thyroid.	0.2 (1 dose)	Dog biscuit, milk.						
	15	11.2	" " "	0.2 (1 ")							

Voit (5)	1	19	Fresh gland.	10 (4 doses)	Meat.	-0.29		6	4	} "
	1	18	" "	10 (2 ")	No food.			25	2	
	1	18	Iodothyron.†	10 (5 ")	Meat.	-1.03		4	5	
	1	18	" "	10 (2 ")	No food.			19	2	
Schöndorff (6)	1	25	Tablets† Fresh gland.	5-10 tablets, 12 gm. fresh gland or 1 gm. dried gland daily.	Horse flesh, 30 gm. N daily.	-0.12		2	24	} Same dog throughout.
	2	25		20 tablets daily.	" "	-1.30		6	23	
	3	25		20 " "	" "	-0.40		3	30	
	4	23		20-30 tablets daily.	" "	Positive.		0	32	
	5	23		20 tablets; 7 gm. thyroiodine; 2 days.		"		0	13	
Georgiewsky (7)	1	3.9	Fresh gland.	20, daily.	Horse flesh, 9 gm.	-0.78		8	5	}
	2	12.7	Cooked thyroid.	50, " "	" " 11 "	-0.69		6	5	
Oswald (8)	1	11.0	Thyreoglobulin.	1 (1 dose)	Dog biscuit and milk.		-2.0	43	1	} Material from goiter of pig.
	2	8.9	" "	1 (1 ")	" " " "		-0.3	6	1	
	3	8.7	" "	1 (1 ")	" " " "		-2.3	55	1	
	4	15.7	" "	1 (1 ")	" " " "		-0.7	9	1	

TABLE I—Concluded.

Experimenter.	Experiment No.	Weight of dog. <i>kg.</i>	Substance.	Dose.	Food.	Average daily N balance. <i>gm.</i>	N balance day of administration.	Average increase in N in urine. <i>per cent</i>	Increase in N on day of administration. <i>per cent</i>	Days of administration.	Remarks.
Underhill and Saiki (10)	1	8.0	Desiccated thyroid.	5, daily.	Meat, cracker meal, lard.	-0.7	-0.8	13	14	7	N in thyroid administered subtracted from N eliminated before calculation is made.
	2	8.0	"	20 (2 doses)	"	-0.1	+0.2	1		2	
	3	7.3	"	50 (1 dose)	"		+1.6			1	
	4	—	"	30 (1 ")	"		-1.0		10	1	
	5	—	"	30 (1 ")	"		-2.1		30	1	

* Consecutive experiments on one dog.

† 1 gm. iodothyron preparation = 1 gm. fresh thyroid gland.

‡ Borroughs and Wellcome tablet = 0.01 N.

§ The nitrogen administered in the thyroid is over 50 per cent of the nitrogen intake, and if not all absorbed in 24 hours could account for no increase in the percentage elimination of nitrogen.

nitrogen. Powdered veal containing 10 to 15 per cent nitrogen was used for the control animals. The 24 hour collections from the metabolism cages, the cage washings, the bladder urine, and the bladder washings were combined and diluted to 2 liters. 5 cc. of diluted urine were taken for nitrogen determinations by the Kjeldahl method and run in duplicate. The records begin after several preliminary collections.

Dog 1,843.

Date.	Dose.	Total N.	Weight.
1918		gm.	kg.
Jan. 12	300 cc. sugar solution daily.	1.7	10.48
" 13		1.6	10.43
" 14		1.6	10.17
" 15		1.5	10.00
" 16	0.5 gm. thyroid.	1.4*	9.97
" 17	0.5 " "	1.6	9.80
" 18	0.5 " "	1.6	9.78
" 19	0.5 " "	1.6	9.58
" 20	0.5 " "	1.7	9.35
" 21	0.5 " "	1.9	9.12
" 22	0.5 " "	1.8	9.04
" 23		1.9	8.93
" 24		1.6	8.84
" 25		1.6	8.78

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 1,847.

Date.	Dose.	Total N.	Weight.
1918		gm.	kg.
Jan. 12	300 cc. sugar solution daily.	1.8	6.97
" 13		1.4	6.91
" 14		1.3	6.83
" 15		1.2	6.74
" 16	0.5 gm. thyroid.	1.3*	6.66
" 17	0.5 " "	1.7	6.57
" 18	0.5 " "	1.7	6.54
" 19	0.5 " "	1.2	6.37
" 20	0.5 " "	1.8	6.23
" 21	0.5 " "	1.9	6.06
" 22	0.5 " "	2.0	6.01
" 23		1.9	5.95
" 24		2.2	5.89
" 25		2.0	5.78

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 1,849.

Date.	Dose.	Total N.	Weight.
<i>1918</i>		<i>gm.</i>	<i>kg.</i>
Mar. 5	200 cc. sugar solution daily.	1.4	10.12
" 6		1.4	9.86
" 7		1.5	9.78
" 8		1.4	9.69
" 9	0.5 gm. thyroid.	1.6*	9.58
" 10		1.8	9.41
" 11		1.8	9.18
" 12		—	9.12
" 13		1.9	9.01
" 14		2.2	8.84
" 15		2.4	8.73
" 16		2.4	—

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 191.

Date.	Dose.	Total N.	Vol. per cent CO ₂ .	Weight.
<i>1918</i>		<i>gm.</i>		<i>kg.</i>
July 6	400 cc. sugar solution daily.	3.6		16.61
" 7		3.6		16.24
" 8		3.4		16.35
" 9		2.9	59.4	16.10
" 10	1 gm. thyroid.	3.0	65.2	15.98
" 11		3.4*	61.3	15.76
" 12		2.4	61.3	15.53
" 13		3.9	59.6	15.19
" 14		4.8	61.3	14.74
" 15		5.3	59.4	14.57
" 16		5.2	59.4	14.23
" 17		5.4	61.4	14.17

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 192.

Date.	Dose.	Total N.	Weight.
1918		gm.	kg.
July 6	300 cc. sugar solution daily.	2.4	11.36
" 7		2.2	11.51
" 8		2.3	11.25
" 9		1.8	11.03
" 10		2.0	10.91
" 11	1 gm. thyroid.	2.2*	10.88
" 12	1 " "	3.4	10.65
" 13	1 " "	2.2	10.65
" 14	1 " "	2.6	10.40
" 15	1 " "	2.5	10.14
" 16	1 " "	2.5	10.03
" 17	1 " "	2.4	9.92
" 18	2 " "	2.2	9.72
" 19	2 " "	2.4	9.69
" 20	3 " "	2.6	9.29
" 21	3 " "	3.6	8.84
" 22	3 " "	4.4	—

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 17, 160.

Date.	Dose.	Total N.	Weight.
1918		gm.	kg.
Aug. 4	400 cc. sugar solution daily.	2.7	22.05
" 5		2.6	21.88
" 6		2.6	21.63
" 7		2.6	21.54
" 8	3 gm. thyroid.	2.6*	21.43
" 9	3 " "	—	21.09
" 10	3 " "	3.6	20.86
" 11	3 " "	4.2	20.63
" 12	3 " "	3.9	20.41
" 13		4.3	20.07
" 14		4.2	19.84
" 15		3.2	19.73
" 16		3.6	19.73
" 17		2.9	19.61

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 1,860.

Date.	Dose.	Total N.	Weight.
<i>1918</i>		<i>gm.</i>	<i>kg.</i>
Aug. 4	400 cc. sugar solution daily.	3.6	22.62
" 5		3.6	22.14
" 6		3.0	22.22
" 7		3.0	21.82
" 8	3 gm. thyroid.	2.9*	21.77
" 9	3 " "	—	21.65
" 10	3 " "	3.6	21.37
" 11	3 " "	4.0	21.03
" 12	3 " "	3.6	20.63
" 13	3 " "	4.4	20.18
" 14		5.4	19.50
" 15		6.0	20.12
" 16		4.8	19.84
" 17		3.1	19.50

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 1,843.

Date.	Dose.	Total N.	Weight.
<i>1917</i>		<i>gm.</i>	<i>kg.</i>
Oct. 12	400 cc. sugar solution daily.	1.5	9.66
" 13		1.3	9.55
" 14		1.3	9.63
" 15	10 gm. thyroid.	1.3*	9.52
" 16	10 " "	1.7	9.38
" 17	10 " "	2.0	9.04
" 18	10 " "	2.1	8.87
" 19	10 " "	2.3	8.56
" 20		2.7	8.27
" 21		2.4	8.16
" 22		2.4	—
" 23		2.0	—

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 1,849, Control.

Date.	Dose.	Total N.	Weight.
<i>1918</i>		<i>gm.</i>	<i>kg.</i>
Jan. 13	300 cc. sugar solution daily.	2.4	8.73
" 14		2.1	8.52
" 15		1.9	8.52
" 16	0.4 gm. meat powder.	1.9	8.38
" 17	0.4 " " "	1.9	8.30
" 18	0.4 " " "	1.9	8.17
" 19	0.4 " " "	1.8	8.15
" 20		1.9	7.95
" 21		1.9	7.86
" 22		2.2	7.75

Dog 1,867, Control.

Date.	Dose.	Total N.	Vol. per cent CO ₂ .	Weight.
<i>1918</i>		<i>gm.</i>		<i>kg.</i>
Mar. 5	300 cc. sugar solution daily.	2.2	53.8	9.29
" 6		2.3	57.4	9.01
" 7		2.5	51.6	8.95
" 8		—		8.84
" 9	0.5 gm. meat powder.	2.1		8.87
" 10	0.5 " " "	1.7		8.78
" 11	0.5 " " "	1.6		8.73
" 12	0.5 " " "	1.4		8.73
" 13	0.5 " " "	1.4		8.61
" 14	0.5 " " "	1.2		8.53
" 15	0.5 " " "	1.3	53.9	8.44
" 16	0.5 " " "	1.3	53.8	—

Dog 1,879, Control.

Date.	Dose.	Total N.	Weight.
<i>1918</i>		<i>gm.</i>	<i>kg.</i>
Jan. 13	300 cc. sugar solution daily.	1.4	6.37
" 14		1.5	6.20
" 15		1.4	6.09
" 16	0.5 gm. meat powder.	1.3	6.01
" 17	0.5 " " "	1.3	5.98
" 18	0.5 " " "	1.2	5.98
" 19	0.5 " " "	1.3	5.92
" 20	0.5 " " "	1.3	5.84
" 21	0.5 " " "	1.3	5.75
" 22	0.5 " " "	1.1	5.69
" 23	0.5 " " "	1.1	5.61
" 24	0.5 " " "	1.2	5.52

* Nitrogen eliminated in the 24 hours preceding the administration of thyroid substance.

Dog 193, Control.

Date.	Dose.	Total N.	Vol. per cent CO ₂ .	Weight.
<i>1918</i>		<i>gm.</i>		<i>kg.</i>
July 5	300 cc. sugar solution daily.	2.5		10.43
" 6		2.4		10.17
" 7		2.3		10.09
" 8		2.3		10.00
" 9		2.1	61.3	9.95
" 10		1.4	55.6	9.69
" 11		2.6	59.4	9.63
" 12	1 gm. meat powder.	2.2	59.4	9.41
" 13	1 " " "	2.2	53.8	9.29
" 14	1 " " "	2.2	59.5	9.24
" 15	1 " " "	2.1	59.5	9.12
" 16	1 " " "	1.2	61.4	9.07
" 17	1 " " "	3.1	61.4	—

Dog 1,859.			Dog 1,821 (control).		
Date.	Dose.	Vol. per cent CO ₂ .	Date.	Dose.	Vol. per cent CO ₂ .
<i>1918</i>			<i>1918</i>		
Aug. 14	336 cc. sugar solution daily.	59.5	Aug. 14	400 cc. sugar solution daily.	57.6
" 15		62.4	" 15		—
" 16		63.3	" 16		58.5
" 17		63.3	" 17		59.5
" 19		63.3	" 19		65.3
" 20		57.6	" 20		58.6
" 21	3 gm. thyroid.	57.7	" 21		59.6
" 22	3 " "	58.6	" 22		59.5
" 23	3 " "	56.6			

DISCUSSION.

These experiments would indicate that studies of the basal nitrogen metabolism of dogs receiving sugar solutions over a period of about 2 weeks is sufficient to indicate whether or not nitrogen metabolism is accelerated by thyroid preparations administered during this period.

The summary of results showing the average nitrogen figures for 3 days preceding thyroid feeding and for the days of administration including two subsequent days,¹ would indicate that a daily dose of 0.05 to 0.1 gm. of desiccated thyroid mixture per kilo of body weight is a dose producing a marked effect upon nitrogen elimination and a somewhat greater percentage loss in weight than is found in the control animals.

The condition of the animals remained excellent throughout the experiments. The carbon dioxide-combining power of the plasma of several of the animals studied was not affected by the thyroid substance given. Fresh thyroid glands were not fed as a control upon these experiments, so at present no discussion of

Summary of Results.

Experiment No.	Dog No.	Dose.	Weight.	Average daily N for 3 days previous to administration.	Average daily N for days of administration + 2 days following.	Increase in N.	Loss in wt. in 3 days before administration.	Loss in wt. in first 3 days of administration.	Loss in wt. in second 3 days of administration.
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Thyroid preparation administered.

		gm. per kg.	kg.	gm.	gm.	per cent	per cent	per cent	per cent
1	1,843	0.05	10	1.5	1.7	13	4.4	3.9	5.6
2	1,849	0.05	10	1.5	2.0	33	2.8	4.8	4.2
3	191	0.07	15	3.1	4.5	45	3.6	6.4	3.8
4	1,847	0.10	6	1.2	1.8	50	3.6	4.3	5.6
5	192	0.1-3	10	2.0	2.8	40	3.2	4.4	4.6
6	17,160	0.15	22	2.6	3.9	50	2.0	3.7	3.3
7	1,860	0.15	22	3.0	4.5	50	1.2	3.4	7.2
8	1,843	1.00	9	1.3	2.2	69	1.4	6.8	8.0

Meat powder administered.

1	1,867	0.05	9	2.3	1.4	Decrease.	1.5	1.6	3.3
2	1,849	0.04	8	1.9	1.9	0	4.0	2.7	4.9
3	1,879	0.1	6	1.4	1.2	Decrease.	5.6	1.5	3.8
4	193	0.1	10	2.0	2.1	4	5.4	3.1	—

¹ The calculations for average percentage increase in nitrogen elimination in the tabular summary of the literature are made on this basis.

the question as to whether the effect of desiccated thyroid upon metabolism is physiological or toxicological can be made.

CONCLUSION.

Nitrogen elimination in the dog receiving only sugar solutions may be increased approximately 50 per cent by the administration, during a 5 to 7 day period, of commercial desiccated thyroid gland in doses of 0.10 to 0.15 gm. per kilo of body weight.

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THE ACID-BASE BALANCE IN ANIMAL NUTRITION.

I. THE EFFECT OF CERTAIN ORGANIC AND MINERAL ACIDS ON THE GROWTH, WELL BEING, AND REPRODUCTION OF SWINE.*

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INTRODUCTION.

The question of the balance between acid- and base-forming mineral elements in foodstuffs has figured more or less prominently in discussions of rations for both man and animals during the last decade. Based principally on the work of Forbes,¹ Sherman and Gettler,² and Kastle,³ there is a tendency to emphasize the necessity for a balance between potential acid and base in the mineral constituents of rations. In this preliminary discussion human dietary standards will figure, as well as rations for animals, but in reporting our conclusions we shall avoid the error of applying data obtained with one species to other species.

Much of the early literature on this subject is cited by Forbes and by Sherman and Gettler. The former's discussion of the acid-base balance is largely theoretical, and much of it is based upon notions which have since been found incorrect. The analyses of Sherman and Gettler, which are the first to show accurately the true relations of the principal mineral elements in foodstuffs, are valuable and their interpretation of their metabolism experiments is conservative, but others have often overemphasized the necessity of a balance such as they suggest. We wish to point out fallacies in the reasoning of those who insist on the necessity of this balance of ash elements.

* Published with the permission of the Director of the Iowa Agricultural Experiment Station. Preliminary report read at the Kansas City meeting of the American Chemical Society, April 12, 1917.

¹ Forbes, E. B., *Ohio Agric. Exp. Sta., Bull.* 207, 1909.

² Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 323.

³ Kastle, J. H., *Am. J. Physiol.*, 1908, xxii, 284.

The interpretation of experiments comparing natural foodstuffs with varying acid and base content is difficult, because the character and composition of the ash, aside from its acid or basic properties, may have much influence on the results, as has been shown by McCollum,⁴ and by Osborne and Mendel.⁵ Other elements in the ration, such as the character of the proteins, may be limiting factors. A prime essential in fundamental nutrition experiments is that the unknown factors as far as possible should be limited to one. Then only is complete interpretation and generalization possible. The tendency has often been to accept too readily as a reason for unfavorable results the potential acid character of the ration, when other possibilities are present. For example, in natural feedingstuffs such as oats, wheat, and corn, the mineral or protein content is often deficient in quality, yet conclusions are sometimes drawn solely on the basis of the excess of acid-forming elements. Weiser⁶ fed pigs on corn alone, which is very low in calcium, obtaining a positive nitrogen and a negative calcium balance. Calcium was added in the form of carbonate and the calcium balance became positive. Funk⁷ implies that this effect obtained by Weiser is due to the correction of the acidity of the ration. In his own work with rabbits Funk finds that a diet of oats alone causes death in 30 to 70 days, and thinks the failure of the ration due to the excess of acid-forming mineral elements in the oats. He, as well as others who have fed rabbits on an exclusive oat diet, appears to disregard the fact that herbivorous animals, including rabbits, require roughage as a constituent of the ration. Unfavorable results from an abnormal as well as incomplete diet are then interpreted as being due to the potential acidity of the oats. It is noteworthy that Funk finds that rats, which are omnivorous, can be maintained on oats and white bread for a long time, although this diet fails to produce growth in young rats. That the failure in growth is not due to the acidity of the diet is shown by the fact that the addition of sodium bicarbonate had no beneficial action. Other cases might be cited from the literature, but the foregoing illustrations show the tendency to consider acidosis as a frequent cause of nutritional troubles.

Acidosis is variously defined. As most generally accepted it is a pathological condition which occurs in diabetes, nephritis, surgical cases, and as a result of poisoning or starvation. In infants it is sometimes a result of too high fat feeding, but it is then clearly due to a limited capacity to digest and assimilate fats. We dislike to use this term, which really belongs to pathology, in discussing normal nutrition. Indeed the widespread tendency

⁴ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.

⁵ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, pt. ii, 1911; *J. Biol. Chem.*, 1918, xxxiv, 131.

⁶ Weiser, S., *Biochem. Z.*, 1912, xlv, 279.

⁷ Funk, C., *J. Biol. Chem.*, 1916, xxv, 409.

to write into the literature of normal nutrition data obtained from drug administration, pathological cases, and other abnormal conditions is to be deprecated. Drug administration is of value, and experiments on diabetic patients are of importance in the study of diabetic acidosis, but unfortunately the data obtained in such studies are widely accepted as showing the limited tolerance of the animal organism for acid, disregarding the fact that the organisms experimented on are in an abnormal or pathological condition.

The protective mechanism of the body against acids has been thoroughly studied by Henderson.⁸ That the organism has considerable tolerance for acid has been shown by McCollum.⁹ He reported satisfactory growth from an early age and normal reproduction in rats fed exclusively on egg yolk, which Sherman and Gettler find to have a potential excess acidity of 26.7 cc. normal solution per 100 gm. McCollum and Hoagland¹⁰ have shown that a pig on a nitrogen-free diet plus mineral acids can neutralize a large part of the acid with ammonia and maintain tissue neutrality. At the same time they found that the creatinine nitrogen in the urine remained constant, suggesting that the extra nitrogen catabolized to neutralize the ingested acid probably originated elsewhere than in muscle tissue. Steenbock, Nelson, and Hart,¹¹ using swine and calves on a normal level of protein intake, found that the rise in urinary ammonia nitrogen was not accompanied by a corresponding rise in the total urinary nitrogen, but that there was a commensurate drop in the urea nitrogen. They concluded that on a normal protein intake the excess of acid-forming mineral elements which might exist in natural foods should exercise no deleterious effect on protein storage.

The possibility is thus established that an animal may be able to grow and complete its normal cycle of life on a ration which contains an excess of acid-forming mineral elements, but which is

⁸ Henderson, L. J., *J. Biol. Chem.*, 1911, ix, 403; *Science*, 1913, xxxvii, 389; 1917, xlvii, 73, and other papers.

⁹ McCollum, E. V., *Am. J. Physiol.*, 1909-10, xxv, 127.

¹⁰ McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 299.

¹¹ Steenbock, H., Nelson, V. E., and Hart, E. B., *J. Biol. Chem.*, 1914, xix, 399.

otherwise satisfactory. It has been shown that animals of all types of feeding habits may divert part of the nitrogen which would otherwise appear in the urine as urea for the production of ammonia, thereby neutralizing a large part of the acids ingested or formed in metabolism. Sherman and Gettler,² however, with a human subject on acid and alkaline diets, found that the extra urinary acidity and ammonia failed to account for all the extra acidity of the acid diet. This fact may be the reason for the tendency to consider a balance between acids and bases necessary. It is possible, however, that the greater part of this remainder may be accounted for in the feces without any serious loss of bases from the body. Inasmuch as bacterial residues constitute a large part of the mass of feces, it is conceivable that some sulfur and phosphorus is excreted combined with protein and in other unoxidized forms. Also, in growth, a considerable amount is stored in the form of proteins, phosphatides, and nuclear material. Thus it seems possible that the excess acid of natural foods may be accounted for without serious loss of base from the body, by means of ammonia production, acid phosphate excretion, and the other factors just mentioned. Of course we refer to *normal* nutrition. The only way to determine definitely whether or not a balance between acid- and base-forming mineral elements is necessary is to test the ability of animals to maintain growth and well being and to reproduce normally on a satisfactory ration with a considerable excess of acid. Inasmuch as swine are convenient experimental animals and grow so rapidly that their weight is quadrupled in a few months, they have been used in our experiments. Swine are generally fed largely on cereals, which always carry an acid ash, and it is important to know for practical reasons whether their rations should be balanced with bases.

EXPERIMENTAL.

These experiments were initiated with the purpose of testing the ability of swine to metabolize successfully the lactic and acetic acids of silage. In the course of our silage investigations at the Iowa station, it has been found possible to make a satisfactorily preserved silage from rape (*Brassica napus*).¹² As or-

¹² Lamb. A. R., and Evvard, J. M., *J. Agric. Research*, 1916, vi, 527.

dinary corn silage is too fibrous to be utilized efficiently by swine, silage has not been well tested in swine feeding. In this experiment lactic, acetic, and sulfuric acids were fed in equivalent amounts to separate lots of pigs, the mineral acid being fed for comparison with the organic acids. Since the lot receiving sulfuric acid grew practically as well as the control lot, the question of the balance of mineral acids and bases was raised, and the work continued on that subject.

The plan of the first experiment was as follows. Eight pigs, all from the same litter, about 100 days old and weighing 50 to 60 pounds, were divided into four lots of two pigs each. All were healthy and thrifty, and similar in appearance and condition. All were fed twice daily the same basal ration, well tried and satisfactory, consisting of 80 per cent ground corn, 15 per cent meat meal tankage (which contained 60 per cent protein), and 5 per cent standard wheat middlings. The amount fed to each lot was regulated by the amount the lot eating least would eat each day. Thus each lot received the same amount of feed—an important matter since the gains in weight of the lots were to be compared. Water in the drinking troughs was allowed *ad libitum*. With this exception the lots were treated similarly in every respect until near the close of the experiment. The mixed feed was mixed with an equal weight of water in the feeding troughs, and the acids, in the form of normal solutions, were thoroughly mixed with the moist feed.

Lot I was used as a control throughout the experiment, Lot II was fed sulfuric acid, Lot III an equivalent amount of lactic acid, and Lot IV an equivalent amount of acetic acid. The total length of the experiment, except for Lot II which was continued longer on sulfuric acid, was seven 30 day periods. The periods and the amounts of acid added to the ration are shown in Table I.

The rate of growth of the four lots was quite uniform in spite of the acid additions. If the growth curves were plotted on a scale small enough to be suitable for reproduction here, it would be difficult to distinguish any difference. Neither growth nor well being appears to have been interfered with appreciably by the acids added to the ration. Table II gives the weights of the animals by 30 day periods. Weights were taken every 10 days. While Lot II weighed considerably less than the control lot at

TABLE I.
Plan of Growth Experiment, Showing Amounts of Normal Acid Fed.

Period No.	Periods. <i>days</i>	Lot I	Lot II	Lot III	Lot IV	Remarks.
I	30 (control)	Basal ration only.	Basal ration only.	Basal ration only.	Basal ration only.	Began Oct. 4, 1916. All lots fed same amount of feed.
II	30	Basal ration only.	100 cc. N sulfuric.	100 cc. N lactic.	100 cc. N acetic.	
III	10	Basal ration only.	200 cc. N sulfuric.	200 cc. lactic.	200 cc. acetic.	
	10	" "	300 " "	300 " "	300 " "	
	10	" "	400 " "	400 " "	400 " "	
IV	30	Basal ration only.	500 cc. sulfuric.	500 cc. lactic.	500 cc. acetic.	
V	30	Basal ration only.	500 cc. acetic.	500 cc. sulfuric.	500 cc. lactic.	Note change in acids.
VI	30	Basal ration only.	500 cc. sulfuric.	500 cc. lactic.	500 cc. acetic.	Lots fed according to appetite last 50 days of experiment.
VII	30 (control)	Basal ration only.	No acid.	No acid.	No acid.	

the close of the experiment, this was partly due to the fact that during the last 50 days all lots were fed according to appetite. It should be noted that at the beginning of Period VI, when all lots were being fed the same, the differences between the lots in average weights were less than the variations between individuals within the lots.

No difficulty was experienced in feeding the acid in the manner described above. At one time only during the experiment, the animals in Lot II lost appetite and did not eat well for a few days. This was soon after being changed from the acetic acid ration back to sulfuric acid. It merely calls attention to the difficulty under

TABLE II.

Weights of Animals at Beginning of Each 30 Day Period and at End of Experiment.

Period.	Lot I		Lot II		Lot III		Lot IV	
	Animal 460	Animal 465	Animal 461	Animal 463	Animal 462	Animal 464	Animal 466	Animal 467
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>
I	66	51	63	55	53	61	59	57
II	95	80	96	80	89	89	77	98
III	127	117	126	114	116	115	105	136
IV	158	157	164	147	157	142	139	176
V	194	197	196	183	199	171	177	216
VI	217	238	238	210	223	200	219	241
VII	260	272	255	244	269	249	247	287
Final weight...	327	327	304	295	303	306	306	336

which these animals labored when they were obliged to take into the stomach 250 cc. of *normal* acid at each feeding time. There was apparently, however, little interference with digestion, as shown by the growth curves. It appears probable that it is at least as difficult for the organism to neutralize acid thus ingested as to neutralize acid produced in the tissues by gradual oxidation. When acid is given by mouth the greater part of it must be neutralized at once by whatever material is most convenient, and readjustments made later. It is possible that this is more wasteful of fixed bases than the neutralization of acid produced gradually in the tissues.

In addition to the superimposed acid, the basal ration itself contained an excess of acid-forming mineral elements amounting to about 2.55 cc. of normal solution per 100 gm. of ration, or about 60 cc. normal solution per pig per day. It should also be noted that the basal ration we fed was nearly as high in potential acidity as would be possible in a satisfactory ration for growing pigs. The amount of acid superimposed upon this ration was more than eight times the amount of the natural potential acidity of the feed. Under these circumstances slight variations in the rate of growth, etc., are of little importance when the interpretation is made from the standpoint of natural rations and their low potential acidity.

TABLE III.
Comparative Data.

	Lot I	Lot II	Lot III	Lot IV
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>
Average initial weight per pig.....	58.6	59.0	57.0	58.3
“ final weight.....	327.4	299.4	304.4	320.7
“ daily gain per pig.....	1.28	1.14	1.18	1.25
“ “ feed.....	5.62	5.26	5.51	5.57
“ “ “ per 100 lbs. live wt.	3.15	3.05	3.24	3.12
“ feed to produce 100 lbs. gain...	439.4	460.0	467.4	445.8
“ daily gain per pig for first five periods only.....	1.13	1.10	1.03	1.14

Some significant comparative data showing the average daily gain, etc., in each lot, are given in Table III. The table shows clearly the relative gains in weight of the four lots. Lot I (control) made a little better gain and somewhat more economically than the others. The record made by Lot I was exceptionally good. Lot II (sulfuric acid) made the least daily gain, but the difference is not very great, perhaps not much greater than variations due to individuality. It should also be noted that Lot II consumed less feed per day and per 100 pounds live weight. All lots received the same amount of feed until the last 50 days, when each lot was allowed as much feed as it would readily consume. Part of the variations shown are due to this change in treatment, since the lot fed sulfuric acid would

not consume so much feed as the others, presumably on account of the admixture of strong acid.

The average daily gain per pig for the first five periods only, when all lots were being fed the same amount of feed, shows more accurately the performance of the various lots than the average for the whole experiment and checks well with other averages for the various periods. It will be noted that the rates of gain in Lots II and III are still a little below Lot IV as well as below the control lot. However, there are other factors to be taken into consideration in the case of Lot II which have already been discussed.

The organic acids fed Lots III and IV were apparently oxidized, as early morning samples of urine taken at intervals showed no higher ammonia content than the urine of the control lot.

TABLE IV.

Animal No.	Lot.	pH
460	I	7.2
465	I	7.1
461	II	7.1
463	II	7.0
464	III	7.1
467	IV	7.2

Growth and well being in these lots were entirely comparable with the control lot. No change in the growth curves was noted when the acids were interchanged in Period V (Table I). Further data on these questions are given in the second paper of this series.

Near the end of the last acid-feeding period, samples of blood were taken from several of the animals and the hydrogen ion concentration was determined by the method of Levy, Rowntree, and Marriott.¹³ The results (Table IV) show definitely that neither the organic nor mineral acids used disturbed the reaction of the blood, and demonstrate the efficiency of the protective mechanism against acids.

¹³ Cf. Hawk, P. B., Practical physiological chemistry, Philadelphia, 5th edition, 1916, 288.

Reproduction Test.

The two animals in Lot II, after the last control period of the preceding experiment (see Table I), and after a 10 day control period during which they were treated for worms (using santonin and calomel) with negative results, were continued on the same ration plus 500 cc. of normal sulfuric acid, just as before. The acid feeding began again on May 12, 1917, and the two animals. Nos. 461 and 463, were bred on April 19 and June 5, respectively. Except for a few feeds during the extremely hot weather these animals ate well, remained in thrifty condition, and each received 500 cc. of normal acid daily.

On August 12, Animal 461 farrowed eight strong vigorous pigs which suckled normally for more than a week, when seven of them died within a day or two. The last one was killed accidentally when nearly a month old. The possibility of some toxic quality in the milk is not excluded, but seems unlikely. We are obliged to consider these results inconclusive.

Animal 463 farrowed three vigorous pigs on September 27. One was transferred to another litter to check if possible the influence of the milk. Unfortunately the other sow killed this pig by lying on it. Of the other two, one died when nearly 4 weeks old. Postmortem examination¹⁴ showed that death was due to pericarditis resulting from infection, which could not be directly ascribed to the ration. The last pig passed successfully through the suckling period, and weighed 33 pounds when 60 days old. The results in this case were more favorable, although not entirely successful. It is apparent, however, that a strongly acid ration did not interfere with prenatal development at least, and the trouble may have been due to some cause outside the ration.

Qualitative tests made on the milk of Sow 463 showed it to be neutral in reaction, normal in appearance and odor, with a trace of sulfates, and a positive test for calcium in the milk serum. It should also be noted that the urine of this sow showed no evidence of glycosuria or albuminuria.

¹⁴ Postmortem examination was made by Dr. W. W. Dimock, Professor of Veterinary Pathology at Iowa State College.

Postmortem examination was not made of the pigs of the first litter, nor was it possible to make slaughter tests of the two sows, nor to examine the character of their bones.¹⁵ The bones of the young pigs, however, were all above the average in size, as there was an abundance of calcium in the meat meal tannage of the ration. This reproduction test will be repeated at our earliest opportunity.

While the results in growth on the sulfuric acid ration were successful and the results in reproduction fairly good, it must be noted that the ration was a good one, containing an abundance of protein and mineral matter, even though the excess of mineral elements was on the acid side. This ration was not better than should be fed to insure good results, but was merely a satisfactory ration. If the ration were lacking in some essential, or poor in quality, the results might be different. These results show, however, that *if the other elements in a natural ration are satisfactory, it is not necessary to balance the acid and basic mineral elements for growing swine*. No suitable combination of natural feeds would contain nearly as much potential mineral acid as was fed in this experiment, during which the animals in Lot II increased in weight from 88 to an average of 370 pounds and successfully produced young.

The metabolism of these acids and the question of the possible loss of fixed bases from the body, are discussed in the second paper of this series.

SUMMARY.

The necessity for balancing the potential acid and base of rations has been in dispute, but nutritional failures are often ascribed to excess acidity when other causes are not wholly excluded.

Four lots of two pigs each were fed equal amounts of a good basal ration. Three of the lots received sulfuric, lactic, and acetic acids respectively, in amounts up to 500 cc. of normal solution per pig per day. This continued for 150 days of acid feeding. The three acid-fed lots grew practically as rapidly as the fourth lot, which was a control, and remained in equally good condition.

¹⁵ Postmortem on a pig fed sulfuric acid in a later experiment showed sternum, ribs, and skull normal. Bones were flinty and hard with less marrow than control.

The organic acids were apparently completely oxidized, and the sulfuric acid was neutralized without apparent harm or significant effect on growth.

The two pigs fed sulfuric acid were continued on the same ration for 4 to 6 months longer and successfully produced young. Either the excessive amount of acid fed or some other factor, however, prevented the successful rearing of the young.

THE ACID-BASE BALANCE IN ANIMAL NUTRITION.

II. METABOLISM STUDIES ON THE EFFECT OF CERTAIN ORGANIC AND MINERAL ACIDS ON SWINE.*

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In Paper I of this series it was reported that swine apparently possess the ability to maintain normal growth and well being when ingesting considerable amounts of lactic, acetic, or sulfuric acids with the ration. This fact has made it necessary to secure more definite information concerning the fate of these acids in the animal body.

While it is known that lactic and acetic acids are oxidizable, several observers have reported traces in normal urine, thus suggesting a limited ability to oxidize them. There is some evidence that lactic acid is an intermediate product in the oxidation of glucose. Embden¹ has shown it to be closely related with alanine and pyruvic acid in the organism. While lactic acid appears in the urine in cases of oxygen starvation, especially in phosphorus poisoning or asphyxiation, it appears under normal conditions to be completely oxidized. Acetic acid is probably completely oxidized, although the mechanism of its oxidation is not known. It appears that these acids should not tax the neutralizing powers of the organism. However, large quantities of these two acids have not hitherto been fed to an animal in a metabolism cage.

In the case of unoxidizable mineral acids, while the mechanism of neutralization has been found to be efficient, various harmful possibilities, such as the withdrawal of fixed bases from the body,

* Published with the permission of the Director of the Iowa Agricultural Experiment Station.

¹ For bibliography on this subject see Dakin, H. D., *Oxidations and reductions in the animal body*, London, 1912.

have been suggested.² The influence of the considerable amounts of ammonium salts formed when acids are neutralized has sometimes been considered detrimental. Voegtlin and King reported³ that intravenous injection of ammonium salts (presumably in dogs) produced symptoms of acid intoxication, which were relieved by the injection of calcium salts. Underhill⁴ also found some evidence of toxicity when considerable amounts of ammonium chloride were given *per os*. The loss of calcium from the bones is the most serious possibility suggested with regard to the fixed bases, which may be used in neutralization. However, misleading conclusions have been drawn on this subject when the experimental animal was receiving a ration too low in calcium for proper nutrition. A single example of this will be cited. Stehle⁵ reports a loss of calcium when hydrochloric acid was administered to a dog. The diet was, however, entirely inadequate in lime, as in several other experiments in the literature where animals were fed on meat alone, without bone. Givens and Mendel⁶ have recently shown that neither acid nor alkali exerts any marked effect on calcium storage. In a later paper Givens⁷ confirms these conclusions.

EXPERIMENTAL.

A vigorous thrifty barrow pig which weighed 60 pounds at 90 days of age at the beginning of this work was placed in a metabolism cage with a separate feeding stall, a modification of the cage used by McCollum. The ration previous to this experiment had been satisfactory. The ration we used during the first cage experiment was the same that was used in the growth experiment recorded in the preceding paper; *viz.*, ground corn 80 per cent, meat meal tankage 15 per cent, wheat middlings 5 per cent. The feed was mixed with water, and the acids were added in the same manner as in the experiment just mentioned. The pig was

² See discussion in Paper I of this series.

³ Voegtlin, C., and King, I., *J. Biol. Chem.*, 1909, vi, p. xxviii.

⁴ Underhill, F. P., *J. Biol. Chem.*, 1913, xv, 327 ff.

⁵ Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.

⁶ Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.

⁷ Givens, M. H., *J. Biol. Chem.*, 1918, xxxv, 241.

fed twice daily. The urine was collected every 24 hours, and the feces by 5 day periods.

The pig was placed in the cage for a preliminary period of 5 days to accustom him to the cage and the ration. The regular experimental periods which followed and the rations with additions are shown in Table I.

TABLE I.

Period No.	Period.	Average daily ration.	Acid fed daily.	Weight of animal.
		<i>lbs.</i>		<i>lbs.</i>
I	Control, 10 days.	1.8	None.	62.0
II	Acid, 10 days.	2.0	250 cc. N lactic.	69.5
III	" 10 "	2.0	300 " " sulfuric.	75.0
IV	" 10 "	2.0	300 " " acetic.	81.0
V	Control, 10 days.	2.0	None.	87.0

Some analytical data on the mixed ration as used are shown in Table II.

TABLE II.

H ₂ O	CaO	MgO	P ₂ O ₅	N
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
11.95	1.12	0.25	1.74	2.85

The urinary acidity was determined by titration with phenolphthalein according to Folin. Urea and ammonia nitrogen were determined by the method of Van Slyke and Cullen. Folin's colorimetric method for creatinine and the Folin-Benedict method for creatine were used. Calcium in urine and feces was determined by McCrudden's method. Total urinary sulfur was determined by Benedict's method, and sulfate sulfur according to Folin.

The analytical data on the urine in the first experiment are given in Table III. Averages of the urinary constituents are given by periods.^a

Since there is always more or less lag in the response of the urinary constituents to changes in the ration, a more nearly cor-

^a Credit is due Mr. W. J. Suer for much of the analytical work reported in Table III.

TABLE III.
Urine Analysis. Experiment I.

Period.	Date of sample.	Volume.	Specific gravity.	Reaction to litmus.	Acidity, 0.1 N.	Total N.	Urea N.	NH ₄ -N	Creati- nine N.	Creatine N.	CaO	Total S.	Sulfate S.	Unoxi- dized S.	Remarks.
I	1917	cc.				gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	Control period.
	June 29	537	1.029	Acid.	—	10.139	7.527	0.977	0.299	—	0.129	0.599	0.549	0.050	
	" 30	922	1.018	"	244	12.023	9.207	1.203	0.338	0.082	0.208	0.770	0.680	0.090	
	July 1	1,770	1.010	Neutral.	186	12.532	9.080	1.675	0.355	0.059	0.183	0.812	0.747	0.065	
	" 2	1,155	1.012	Acid.	295	11.573	8.384	1.588	0.343	0.000	0.318	0.714	0.666	0.048	
	" 3	830	1.016	"	282	9.213	6.015	1.459	0.293	0.040	0.267	0.619	0.575	0.044	
	" 4	640	1.020	"	237	8.448	5.720	1.663	0.267	0.096	0.227	0.557	0.509	0.048	
	" 5	525	1.024	"	304	7.718	4.966	1.414	0.286	0.107	0.171	0.516	0.469	0.047	
	" 6	560	1.024	"	319	10.248	7.259	1.443	0.301	0.108	0.199	0.632	0.565	0.067	
	" 7	640	1.021	"	294	10.957	7.974	1.423	0.321	0.064	0.173	0.612	0.533	0.079	
	" 8	690	1.025	Neutral.	262	10.805	7.798	1.329	0.325	0.156	0.196	0.690	0.505	0.085	
Average....		827	1.020		270	10.37	7.39	1.42	0.313	0.079	0.207	0.651	0.589	0.062	Began 200 cc. N lactic daily.
II	July 9	780	1.022	Acid.	288	11.684	8.509	1.332	0.336	0.133	0.221	0.632	0.573	0.059	
	" 10	795	1.021	"	294	10.653	7.509	1.560	0.332	0.137	0.258	0.647	0.559	0.088	
	" 11	605	1.025	"	314	9.995	6.994	1.320	0.308	0.226	0.231	0.649	0.582	0.057	
	" 12	540	1.028	"	227	10.130	7.082	1.409	0.270	0.121	0.238	0.534	0.513	0.021	
	" 13	595	1.025	"	321	10.032	7.338	1.215	0.338	0.086	0.274	0.620	0.563	0.057	
	" 14	575	1.029	"	282	10.212	7.686	1.079	0.346	0.096	0.218	0.660	0.580	0.080	
	" 15	540	1.028	"	227	10.217	7.597	1.146	0.319	0.042	0.219	0.591	0.548	0.043	
	" 16	543	1.029	"	282	10.208	7.504	1.236	0.308	0.024	0.213	0.503	0.468	0.035	
	" 17	562	1.029	"	337	10.487	7.532	1.342	0.313	0.098	0.243	0.628	0.554	0.074	
	" 18	640	1.028	"	346	10.918	7.946	1.122	0.356	0.123	0.218	0.684	0.500	0.094	
Average....		617	1.026		292	10.45	7.54	1.28	0.323	0.109	0.233	0.615	0.553	0.061	Began 300 cc. N lactic.
	July 9	780	1.022	Acid.	288	11.684	8.509	1.332	0.336	0.133	0.221	0.632	0.573	0.059	
	" 10	795	1.021	"	294	10.653	7.509	1.560	0.332	0.137	0.258	0.647	0.559	0.088	
	" 11	605	1.025	"	314	9.995	6.994	1.320	0.308	0.226	0.231	0.649	0.582	0.057	
	" 12	540	1.028	"	227	10.130	7.082	1.409	0.270	0.121	0.238	0.534	0.513	0.021	
	" 13	595	1.025	"	321	10.032	7.338	1.215	0.338	0.086	0.274	0.620	0.563	0.057	
	" 14	575	1.029	"	282	10.212	7.686	1.079	0.346	0.096	0.218	0.660	0.580	0.080	
	" 15	540	1.028	"	227	10.217	7.597	1.146	0.319	0.042	0.219	0.591	0.548	0.043	
	" 16	543	1.029	"	282	10.208	7.504	1.236	0.308	0.024	0.213	0.503	0.468	0.035	
	" 17	562	1.029	"	337	10.487	7.532	1.342	0.313	0.098	0.243	0.628	0.554	0.074	
	" 18	640	1.028	"	346	10.918	7.946	1.122	0.356	0.123	0.218	0.684	0.500	0.094	
Average....		617	1.026		292	10.45	7.54	1.28	0.323	0.109	0.233	0.615	0.553	0.061	

III	July 19		970	1.022	Acid.	310	9.603	5.767	2.140	0.313	0.097	0.393	2.564	2.388	0.176	Began 300 cc. N H ₂ SO ₄ daily.
	"	20	725	1.032	"	370	9.541	6.110	2.424	0.312	0.040	0.612	3.961	3.666	0.295	
IV	Average . . .	"	21	1.145	"	332	10.534	5.463	3.142	0.382	0.026	0.670	4.128	3.813	0.315	
		"	22	810	1.030	437	11.259	6.138	3.252	0.406	0.021	0.853	4.269	4.002	0.267	
		"	23	950	1.028	437	11.647	6.118	3.469	0.402	0.000	0.957	4.694	4.464	0.230	
		"	24	885	1.027	416	11.293	5.680	3.558	0.410	0.014	0.950	4.446	4.387	0.059	
		"	25	1,345	1.021	390	12.912	6.154	4.196	0.414	0.110	0.986	5.067	4.627	0.440	
		"	26	995	1.026	507	12.139	6.394	4.296	0.415	0.120	0.773	5.386	4.544	0.842	
		"	27	740	1.027	340	9.235	4.914	3.498	0.349	0.071	0.259	3.632	3.404	0.228	
		"	28	1,340	1.024	456	15.812	7.076	6.566	0.552	0.169	0.433	6.098	5.494	0.604	
		Average . . .	991	1.026	—	399	11.40	5.98	3.65	0.396	0.067	0.689	4.425	4.079	0.346	
		July 29	1,200	1.020	Acid.	408	12.648	6.498	4.657	0.467	0.107	0.296	3.133	2.765	0.368	Began 300 cc. N acetic daily.
		"	30	1,510	"	242	10.691	5.886	3.822	0.314	0.140	0.109	0.867	0.687	0.180	
		"	31	1,635	"	—	10.693	6.181	2.197	0.370	0.212	—	0.800	0.623	0.177	
		Aug. 1†	—	—	—	—	—	—	—	—	—	—	—	—	—	
		"	2	858	Acid.	12.441	8.048	2.360	0.344	0.344	0.254	0.391	0.922	0.685	0.237	
		"	3	1,005	"	12.080	7.904	2.136	0.324	0.324	0.302	0.290	0.635	0.505	0.130	
		"	4	1,080	"	11.426	7.212	1.678	0.397	0.397	0.412	0.219	0.660	0.574	0.086	
		"	5	845	"	11.204	7.370	1.488	0.288	0.288	0.210	0.171	0.607	0.474	0.133	
		"	6	650	"	10.894	7.404	1.478	0.311	0.311	0.051	0.153	0.737	0.625	0.112	
		"	7	885	"	12.408	8.242	1.601	0.355	0.355	0.236	0.181	0.721	0.578	0.143	
		Average . . .	1,074	1.019	—	11.61	7.19	2.38	0.352	0.352	0.214	0.226	1.009	0.835	0.174	

TABLE III—Concluded.

Period.	Date of sample.	Volume.	Specific gravity.	Reaction to litmus.	Acidity, 0.1 N.	Total N.	Urea N.	NH ₄ -N	Creatinine N.	Creatinine N.	CaO	Total S.	Sulfate S.	Unoxidized S.*	Remarks.
	1917	cc.			cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
V	Aug. 8	720	1.025	Acid.		13.032	9.001	1.482	0.433	0.120	0.226	0.617	0.524	0.093	Control period.
	" 9	812	1.023	"		10.848	6.921	1.582	0.383	0.171	0.280	0.721	0.606	0.115	
	" 10	857	1.020	"		10.181	6.591	1.385	0.362	0.144	0.130	0.649	0.547	0.102	
	" 11	905	1.021	"		10.245	6.614	1.444	0.339	0.175	0.145	0.701	0.603	0.098	
	" 12	713	1.023	"		9.982	6.009	1.697	0.278	0.135	0.123	0.666	0.580	0.086	
	" 13	633	1.027	"		10.836	7.129	1.379	0.294	0.147	0.102	0.657	0.557	0.100	
	" 14	957	1.019	"		11.867	7.608	2.039	0.337	0.128	0.080	0.760	0.652	0.108	
	" 15	710	1.023	"		10.465	6.799	1.630	0.296	0.223	0.068	0.670	0.571	0.099	
	" 16	540	1.024	"		10.214	7.141	1.178	0.271	0.155	0.101	0.619	0.552	0.067	
	" 17	620	1.024	"		11.172	7.635	1.601	0.306	0.080	0.111	0.711	0.612	0.099	
	Average	747	1.023			10.88	7.15	1.54	0.330	0.148	0.137	0.677	0.581	0.097	

All data on 24 hour output.

* By difference.

† Part of sample lost.

rect average for each period has been obtained by taking the last 8 days of each 10 day period. These figures are shown in Table IV.

It will be noted that neither the lactic nor the acetic acid (Periods II and IV) caused any marked increase in the ammonia nitrogen excreted, although it is a little higher in Period IV. The variations in water intake, since this experiment was carried on during the summer, have caused considerable variation in the volume of urine and the analytical data. When the urinary volume, total nitrogen, creatinine, and creatine are plotted on the

TABLE IV,
Urinary Constituents. Daily Average of Last 8 Days of Each Period.

	Period.				
	I, control.	II, lactic.	III, sulfuric.	IV, acetic.	V, control.
Volume, cc.	851	575	1,026	994	742
Specific gravity.....	1.019	1.028	1.026	1.019	1.023
Acidity, 0.1 N, cc.....	272	292	414	—	—
Total N, gm.....	10.19	10.28	11.85	11.59	10.62
Urea N, gm.....	7.15	7.46	5.99	7.48	6.94
NH ₃ -N, gm.....	1.50	1.24	4.00	1.85	1.54
Creatinine N, gm.....	0.311	0.320	0.416	0.341	0.310
Creatine N, gm.....	0.079	0.102	0.066	0.240	0.148
CaO, gm.....	0.217	0.232	0.735	0.234	0.108
Total S, gm.....	0.643	0.609	4.715	0.740	0.679
Sulfate S, gm.....	0.582	0.550	4.342	0.581	0.584
Unoxidized S, gm.....	0.060	0.058	0.373	0.145	0.095

same sheet, it is evident that many of the variations in these constituents are due to the changes in the water intake and urine volume. The higher water intake during Period IV thus accounts for a slight increase in urinary ammonia. Daily tests for either lactic or acetic acid in the urine of their respective periods were always negative. It is probable, therefore, that these acids were quite completely oxidized.

In Period III the addition of sulfuric acid to the ration caused a large increase in the urinary ammonia, the greater part of which is compensated for by the decrease in urea excreted. It is noteworthy that the creatinine excretion is somewhat higher, although

McCollum and Hoagland found no increase in the case of a pig on a nitrogen-free diet.⁹ It may also be noted that the creatine nitrogen is no higher in Period III than in Period I. The extra creatine excretion in the two organic acid periods may or may not be accidental.

The nitrogen balance for this experiment is given in Table V. It shows plainly that the large amount of sulfuric acid ingested did not interfere with nitrogen storage, as was demonstrated with swine and calves by Steenbock, Nelson, and Hart.¹⁰

Of the extra acidity ingested, *viz.* 300 cc. normal solution, 184 cc. are accounted for by the increased ammonia excretion. Apparently only 14 cc. are excreted as extra urinary acidity. This leaves about 100 cc. of normal solution unaccounted for. As

TABLE V.
Nitrogen Balance by Periods. Experiment 1.

Period.	N intake.	N in feces (dried).	N in urine.	Total excreted.	N balance.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
I, control.....	235.91	65.81	103.66	169.47	+66.44
II, lactic.....	259.24	71.72	104.54	176.26	+82.98
III, sulfuric.....	259.24	61.17	113.97	175.14	+84.10
IV, acetic.....	259.24	57.88	116.09	173.97	+85.27
V, control.....	259.24	69.34	108.84	178.18	+81.06

suggested in the preceding paper the greater part of this may be excreted in the feces, either combined with bases or in some other form.¹¹ The calcium balance by periods is shown in Table VI, which apparently accounts for 25 cc. of normal solution of acid daily.

The extra excretion of calcium in the feces in Period III, decreasing the positive balance, may have been due to the formation of calcium sulfate in the intestine during the early stages of digestion, most of which, on account of its relative insolubility, was

⁹ McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 314.

¹⁰ Steenbock, H., Nelson, V. E., and Hart, E. B., *J. Biol. Chem.*, 1914, xix, 399.

¹¹ A complete mineral analysis of the feces was planned but could not be carried out, because of present conditions.

excreted as such in the feces. We have already suggested that the neutralization of acid fed by mouth might be more wasteful of fixed base than the neutralization of an equivalent amount of acid formed in normal metabolism, and this observation agrees with that hypothesis. Givens and Mendel¹² found that the administration of 1.5 gm. of hydrochloric acid per day to a dog caused a diversion of calcium from the intestinal path of excretion to the urine, with no change in the calcium balance. Our results (Table VI) show the increase in urinary calcium, but another increase instead of a decrease in the fecal calcium. It is probably due to the difference in solubility between the chloride and sulfate of calcium, and also agrees with our hypothesis. Therefore the loss of calcium shown in the table may be due to the char-

TABLE VI.
Calcium Balance. Experiment 1.

Period.	Ca intake, CaO.	Ca in feces, CaO.	Ca in urine, CaO.	Total excreted.	Balance by periods.	Average daily balance.
	gm.	gm.	gm.	gm.	gm.	gm.
I, control.....	92.54	57.64	2.07	59.71	+32.83	+3.28
II, lactic.....	101.70	65.15	2.33	67.48	+34.22	+3.42
III, sulfuric.....	101.70	69.03	6.89	75.92	+25.78	+2.58
IV, acetic.....	101.70	63.59	2.30	65.89	+35.81	+3.58
V, control.....	101.70	67.53	1.37	68.90	+32.80	+3.28

acter of the acid and the method of administration. One would certainly not be justified in assuming that if it were possible to find a wholly satisfactory ration containing a potential mineral acidity equal to the amount of acid we fed, such a ration would cause the decrease in positive calcium balance we have noted here. While the calcium in the ration was not more abundant than animal husbandry experience would justify for practical feeding, it was high enough to cause satisfactory storage of calcium. In our second experiment on a ration low in calcium, the animal was not so wasteful of lime in neutralizing the acid fed, but neutralized a larger proportion with ammonia and phosphates.

It is interesting to note that the lactic and acetic acids in Periods II and IV apparently caused an increased storage of

¹² Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 428.

lime. The following explanation seems possible. Perhaps the soluble calcium salts of lactic and acetic acids were absorbed and taken to the tissues where the acid radical was oxidized, thus leaving the calcium in a more favorable position for retention. The fact that during these two periods the urinary calcium was also increased over the control periods also makes it appear that the absorption of calcium from the tract in the form of these soluble salts was increased, with consequent increase both in calcium storage and in urinary calcium.

The hydrogen ion concentration of the blood was again determined on the last day of the sulfuric acid period and found to be 7.2.

Low Calcium Ration.

The object of the second cage experiment was to determine the effect of mineral acid on the calcium balance using a ration very low in calcium, and also to check the urine analysis on a constant water intake. This experiment was carried on in October in a uniformly heated room, and it was intended to allow a definite amount of water throughout the experiment. The feeder failed to allow extra water during the last period to compare with the volume of acid solution given in Period II, and the urine volume dropped unusually low in Period III. More uniform daily results were obtained, however, in Periods I and II.

The ration used was 95 per cent of ground corn and 5 per cent of "black albumin," a blood product low in calcium, but containing over 88 per cent of protein. The calcium content of the mixed feed was 0.022 per cent, and the nitrogen 2.04 per cent, or calculated as crude protein, 12.75 per cent. This was a satisfactory ration except for its very low content of lime.

The analytical methods were the same as used in the first experiment, except that inorganic sulfates were determined instead of total sulfates, which include a small amount of ethereal sulfates. The results of the urine analysis are given in Table VII.

Averages by periods are shown in Table VIII, the first 2 days of each period having been omitted in calculating the averages for reasons previously mentioned.

It will be noted at once that the increase in ammonia excretion is greater than in the first experiment for the same amount of

Urine Analysis. Experiment 2.

Period.	Date of sample.	Volume.	Specific gravity.	Reaction to litmus.	Acidity, 0.1 N.	Total N.	Urea N.	NH ₄ -N	Creatinine N.	Creatinine N.	CaO	Inorganic sulfates as H ₂ SO ₄ .	Remarks.
I	1917	cc.			cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
	Oct. 22	780	1.017	Neutral.	164	8.797	6.118	1.631	0.411	—	0.086	—	Control period.
	" 23	1,145	1.016	Acid.	355	11.301	7.896	1.940	0.513	0	0.098	—	
	" 24	1,160	1.016	"	383	11.433	8.088	1.656	0.536	0	0.118	—	
	" 25	1,050	1.019	"	378	12.524	9.275	1.485	0.526	0	0.086	—	
	" 26	820	1.020	"	271	10.137	8.059	1.217	0.456	0	0.079	—	
Average . . .		991	1.018		310	10.84	7.89	1.59	0.488	0	0.093	—	
II	Oct. 27	1,440	1.020	Acid.	374	12.197	8.225	2.339	0.618	0	0.317	10.359	Wt. of animal, 137 lbs.
	" 28	1,005	1.030	"	543	10.533	6.228	2.856	0.431	0.007	0.435	14.354	
	" 29	1,320	1.025	"	673	12.567	5.692	4.657	0.661	0.102	0.443	15.114	
	" 30	1,165	1.029	"	804	11.883	5.676	4.632	0.357	0.388	0.475	14.015	300 cc. N H ₂ SO ₄ daily with feed.
	" 31	965	1.030	"	627	12.132	5.500	4.550	0.284	0.568	0.670	13.905	
	Nov. 1	915	1.032	"	540	10.607	5.047	4.074	0.339	0.348	0.587	12.925	
III	" 2	1,190	1.024	"	666	12.678	5.964	4.698	0.447	0.149	0.688	13.671	
	" 3	1,155	1.028	"	762	13.405	6.170	4.916	0.358	0.555	0.681	14.550	
	" 4	1,030	1.028	"	670	10.997	4.695	4.557	0.364	0.268	0.611	13.421	
	" 5	1,090	1.029	"	730	13.368	5.890	5.219	0.268	0.460	0.747	15.540	
	Average . . .	1,127	1.028		639	12.04	5.91	4.25	0.413	0.285	0.565	13.785	
	Nov. 6	780	1.028	Acid.	679	14.218	6.813	3.189	0.249	0.337	0.352	4.667	Wt. of animal, 153 lbs.
	" 7	750	1.029	"	660	11.970	7.268	2.952	0.425	0.219	0.188	2.546	Control period.
	" 8	590	1.033	"	513	10.209	6.456	1.817	0.296	0.295	0.119	1.882	
	" 9	585	1.035	"	673	11.029	6.929	1.785	0.288	0.279	0.153	2.038	
	" 10	440	1.036	"	484	8.399	5.605	1.220	0.186	0.192	0.063	1.394	
Average . . .		629	1.032		602	11.17	6.61	2.07	0.289	0.264	0.175	2.505	

acid intake. In the first experiment the per cent of urinary nitrogen as ammonia was 14.7 per cent in the first control period and 33.8 per cent in the sulfuric acid period. In the second experiment the figures were 12.8 per cent in the first control period and 38.2 per cent in the acid period. This may be due to the fact that there was very little calcium available to hold part of the sulfuric acid in the intestine as calcium sulfate. In this experiment 96 per cent of the sulfuric acid ingested was excreted in the urine, while in the first experiment but 90 per cent appeared in the urine, including the ethereal sulfates.

In this experiment there was an apparent decrease in creatinine excretion during the acid period, and a marked increase in

TABLE VIII.

Urinary Constituents. True Daily Averages by Periods. Experiment 2.

	Periods.		
	I, control.	II, sulfuric.	III, control.
Volume, cc.....	1,010	1,104	538
Specific gravity.....	1.018	1.028	1.034
Acidity, 0.1 N, cc.....	377	684	557
Total N, gm.....	11.37	12.21	9.88
Urea N, gm.....	8.47	5.58	6.33
NH ₃ -N, gm.....	1.45	4.66	1.61
Creatinine N, gm.....	0.506	0.385	0.257
Creatine N, gm.....	0.0	0.355	0.255
CaO, gm.....	0.094	0.613	0.112
Inorganic sulfates as H ₂ SO ₄ , gm....	—	14.143	1.771

creatinine. There are too many other possible factors concerned in the excretion of these substances to allow an explanation with the data at hand.

The extra ammonia excreted during the acid period accounts for 229 cc. of the 300 cc. normal solution of acid ingested. The extra urinary acidity accounts for 31 cc., leaving only 40 cc. not taken care of by these two factors in the urine. The calcium balance data (Table IX) show that the difference is not made up by calcium excretion, although there is a slight increase in the negative calcium balance over the average of the two control periods. The difference between the balances in the two control periods is due to the fact that the experimental animal had been

fed the high calcium ration of our first experiment until 5 days before the beginning of the first control period of this experiment. The preliminary 5 day period was not long enough to get rid of what might be considered excess calcium in the tissues of the animal. Therefore for comparison with the acid period it is necessary in this case to take the average of the two control periods. The negative calcium balance throughout is of course due to the inadequate supply of lime in the ration. It should be noted that the extra calcium excretion in the acid period is very

TABLE IX.

Calcium Balance. Experiment 2.

Period.	Ca intake, CaO.			Ca in feces, CaO.	Ca in urine, CaO.	Total CaO excreted.	Ca balance, CaO.	Average daily balance, CaO.
	Feed.	Water.	Total.					
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
I, control, 5 days....	1.50	0.83	2.33	5.47	0.46	5.93	-3.60	-0.72
II, H ₂ SO ₄ , 10 "	3.00	1.56	4.56	5.84	5.65	11.49	-6.93	-0.69
III, control, 5 "	1.50	0.79	2.29	2.12	0.88	3.00	-0.71	-0.14

TABLE X.

Nitrogen Balance. Experiment 2.

Period.	N intake.	N in feces (dried).	N in urine.	Total N excreted.	N balance (by periods).	Average daily N balance.
	gm.	gm.	gm.	gm.	gm.	gm.
I, control, 5 days.....	138.92	22.13	54.19	76.32	+62.60	+12.52
II, H ₂ SO ₄ , 10 "	277.85	30.98	120.37	151.35	+126.50	+12.65
III, control, 5 "	138.92	18.70	55.82	74.52	+64.40	+12.88

small, and that on a low calcium ration the animal was much more economical with the calcium supply than in the first experiment. Even on the high calcium ration, however, only 5 per cent of the very high acid intake was apparently neutralized by calcium, and this may have been largely due to the insolubility of the salt which was the product of neutralization. Although stress has been laid on the effect of natural acid rations on the skeleton, an inspection of the two calcium balance tables shows that the loss caused by the very high amount of acid we fed was

not in either case serious, especially in the first experiment, when the ration was adequate to meet the normal needs of the growing animal.

The nitrogen balance is shown in Table X. As in the first experiment there was certainly no interference by the acid with nitrogen storage.

We intend to continue this work later, to determine, if possible, the fate of the fraction of the acid not yet accounted for.

SUMMARY.

Metabolism studies on a growing pig were conducted to secure definite information regarding the fate of ingested lactic, acetic, and sulfuric acids in the animal body.

On a ration containing a liberal allowance of calcium it was found that the animal apparently oxidized the organic acids completely, with no increase in urinary ammonia, and that they seemed to bring about a slightly increased retention of calcium.

On the same basal ration plus 300 cc. of normal sulfuric acid per day, 61 per cent of the acid ingested was neutralized by means of ammonia, and 5 per cent excreted as phosphates. On another basal ration very low in calcium, extra ammonia excretion accounted for 76 per cent of the acid fed, and extra urinary acidity for 10 per cent. On neither ration did the mineral acid cause a significant loss of calcium, nor did it interfere with the storage of protein.

THE EFFECT OF ACETONE AND OF β -HYDROXYLBUTYRIC AND ACETOACETIC ACIDS ON THE BLOOD CATALASE.

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(Received for publication, January 10, 1919.)

Of the great number of investigators (1) who have worked on the respiratory metabolism in diabetes some have found a decrease in metabolism, some an increase, while others have found no change from the normal. Benedict and Joslin (2) have carried out the most extensive investigations of all those who have worked on diabetes in man and found an increase of about 15 per cent in metabolism. Benedict suggests that this increase in oxidation may be due to the stimulation of the cells of the body by the accumulation of substances such as β -hydroxybutyric and acetoacetic acids and acetone. From the literature it would seem that the preponderance of evidence is in favor of those who claim that oxidation is increased in diabetes.

We had already found that when oxidation was increased, as for example after the ingestion of food, there resulted an increase in catalase due to the stimulation of its production by the alimentary glands, particularly the liver, and that when oxidation was decreased, as in chloroform narcosis, there resulted a corresponding decrease in catalase due to the direct destruction and decreased output of this enzyme from the liver. These and similar observations were taken to mean that catalase is the enzyme in the body principally responsible for oxidation. The present investigation was begun in an attempt to determine whether the introduction into animals of acetone and β -hydroxybutyric and acetoacetic acids, substances which are found in large quantities in severe cases of diabetes, would stimulate the alimentary glands, particularly the liver, to an increased output of catalase, and thus produce an increase in oxidation. The animals used were rab-

bits and dogs. The β -hydroxylbutyric and acetoacetic acids were given in the form of sodium salts. The amounts of the substances will be given in the description of the individual experiments. The catalase was determined by adding 0.5 cc. of blood to diluted hydrogen peroxide at approximately 22°C. in a bottle, and the amount of gas liberated in 10 minutes was taken as a measure of the amount of catalase in the 0.5 cc. of blood.

After exposing the jugular vein and opening the abdominal wall with the use of a local anesthetic, quinine and urea hydrochloride, 5 gm. per kilo of 30 per cent solutions of acetone and β -hydroxylbutyric and acetoacetic acids were introduced into the upper part of the small intestines of rabbits. Determinations of the catalase of blood taken from the jugular were made before as well as at 15 minute intervals after the introduction of the materials. The results of the determinations are given in Fig. 1. The ordinates represent amounts of catalase measured in cc. of oxygen, and the abscissæ, time in minutes. It may be seen under acetone that previous to the introduction of this substance into the intestine, 0.5 cc. of blood liberated 285 cc. of oxygen from hydrogen peroxide; 30 minutes after the introduction, 0.5 cc. of blood liberated 350 cc. of oxygen, and after 45 and 60 minutes, it liberated 360 cc. of oxygen. It may be seen also under acetoacetic acid and β -hydroxylbutyric acid that the introduction of these substances into the intestine produced an increase in catalase.

The object of the second part of this paper was to determine the mode of action of acetone and β -hydroxylbutyric and acetoacetic acids in producing an increase in catalase. We had already observed that the catalase content of the blood of the liver was always 15 to 20 per cent higher than that of the blood from any other part of the body. This observation was interpreted to mean that the liver was continually replenishing the blood and hence the tissues with catalase. We had also found that after the ingestion of food the catalase of the blood of the liver was increased more rapidly than that from any other part of the body. This observation was taken to mean that the absorbed food materials, being carried to the liver, were stimulating this organ to an increased output of catalase. It was also found that when the liver was cut out of the circulation by means of an Eck fistula and by tying off the hepatic arteries, the introduction

of ethyl alcohol into the alimentary tract produced a very small or no increase, whereas normally this substance produces a large increase in catalase. From these observations, the conclusion was drawn that the liver is the principal organ in which catalase is formed and given off to the blood. Stated more specifically,

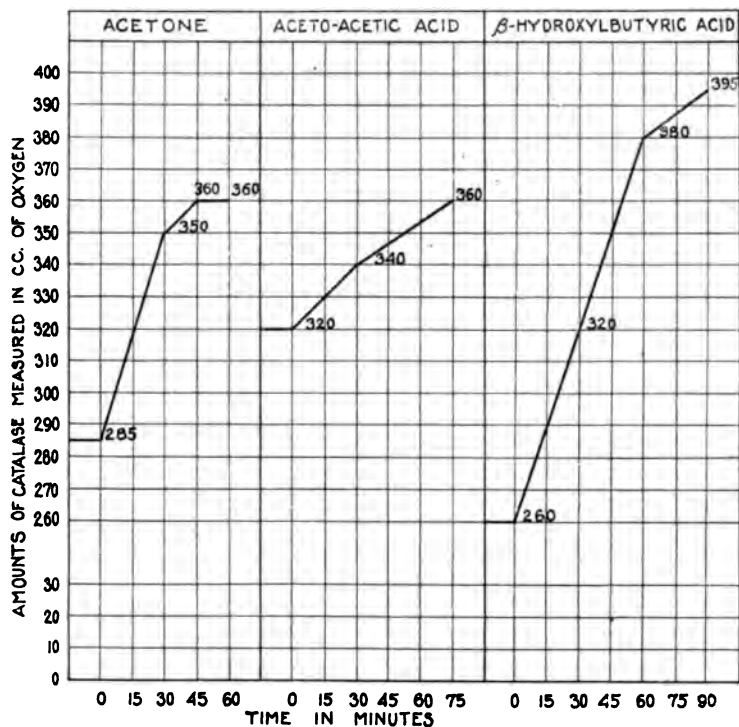


FIG. 1. Curves showing the increase produced in the catalase of blood of rabbits by the introduction into the intestine of acetone, β -hydroxylbutyric acid, and acetoacetic acid.

the second part of this paper is concerned with determining whether the increase in catalase after the introduction of acetone and β -hydroxylbutyric and acetoacetic acids is due to the stimulation of the liver to an increased output of this enzyme.

After etherizing dogs and opening the abdominal wall, 5 gm. per kilo of 30 per cent solution of acetone, β -hydroxylbutyric acid,

and acetoacetic acid were introduced into the upper part of the intestine. The catalase in 0.5 cc. of blood taken directly from the liver and the portal and jugular veins was determined before as well as at 15 minute intervals after the introduction of the

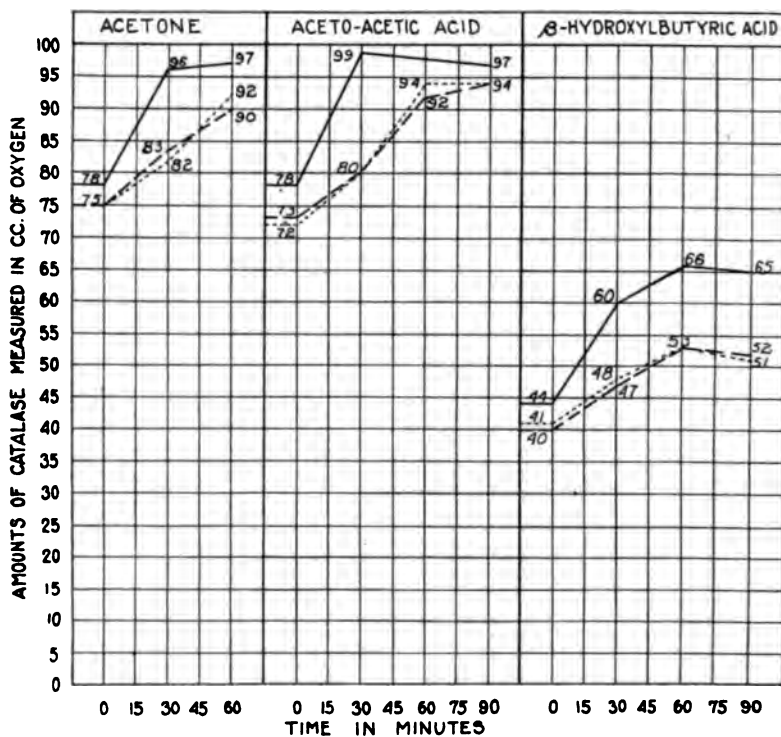


FIG. 2. Curves showing the increase in the catalase of the blood of the liver and portal and jugular veins produced by the introduction of acetone and β -hydroxybutyric and aceto-acetic acids into the intestine of dogs. The solid line curves show amount of catalase in the blood of the liver, broken line curves the amount in the portal vein, and the dotted line curves the amount in the blood of the jugular vein.

different materials. The blood of the liver was collected from a superficial incision made in this organ. The results of the determinations are given in Fig. 2. The ordinates represent amounts of catalase measured in cc. of oxygen, and the abscissæ, time in minutes. The solid line curves were constructed from data ob-

tained from the blood of the liver, the broken line curves from the blood of the portal vein, and the dotted line curves from the blood of the jugular vein. It may be seen under acetone that previous to the introduction of this material into the intestine, 0.5 cc. of blood from the liver liberated 78 cc. of oxygen from hydrogen peroxide, while 0.5 cc. of blood from the portal and jugular veins liberated 75 cc. 30 minutes after the introduction of the acetone, the catalase of the blood of the liver and the portal and jugular veins was increased as indicated by the increase in the amount of oxygen liberated from hydrogen peroxide. The catalase of the blood of the liver was increased particularly during the first 15 minutes to a greater extent than that of the portal or jugular veins. This is taken to mean that the acetone was stimulating the liver to an increased output of catalase. It may be seen also that the introduction into the intestine of the acetoacetic and β -hydroxylbutyric acids produced an increase in catalase.

SUMMARY.

The increased oxidation in diabetes is attributed to the increase in catalase which is due to the stimulation of the liver to an increased output of this enzyme by acetone and β -hydroxylbutyric and acetoacetic acids.

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2. Benedict, F. G., and Joslin, E. P., *Carnegie Institution of Washington. Publication 138*, 1910; *Publication 176*, 1912.



LACTOSE, FAT, AND PROTEIN IN MILK OF VARIOUS ANIMALS.

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During the past year we published descriptions of two methods¹ for the determination of lactose in milk, by means of which we were able to determine the sugar in milk rapidly, and with a considerable degree of accuracy. These methods have also the advantage that they may be carried out on relatively small quantities of material, a factor of importance when the milk of small animals is to be studied. In Tables I to III we have collected the results obtained by the use of our method in the examination of the milk of various animals. For the sake of completeness we have also included the figures for fat and protein.

Fat was determined by the Babcock method for the larger animals, while in the guinea pig, the rabbits, the cats, and the dog we have employed Bloor's nephelometric method² which requires but 1 cc. of milk for duplicate determinations. Protein was calculated from the nitrogen figures obtained by the Kjeldahl-Gunning method.

The figure given for cow's milk is an average of the analysis of the milk of twenty-one cows from the Hospital dairy; these cows included eleven Holstein, four Jersey, and six of mixed breed.

We are indebted to Dr. Fritz Talbot for the samples of milk of the goat, sheep, dog, mare, and women.

The data obtained on the cats and rabbits cover practically the entire period of active lactation, as we discontinued milking these animals only when the amount of milk obtainable was so small as to be inadequate for analysis. Our cats were fed on lean beef and whole milk, the rabbits on oats and cabbage.

¹ Folin, O., and Denis, W., *J. Biol. Chem.*, 1918, xxxiii, 521.

² Bloor, W. R., *J. Am. Chem. Soc.*, 1914, xxxvi, 1300.

TABLE I.
Analyses of Rabbit's Milk.

Date of milking.	Lactose.	Fat.	Protein.	Remarks.
1918	per cent	per cent	per cent	
Mar. 23	1.45	8.7	13.75	Rabbit 1. Delivered Mar. 12.
" 25	2.00	8.4	14.3	
" 29	2.00	6.3	10.1	
Apr. 1	1.60	15.3	11.8	Very little milk. Young rabbit practically weaned.
" 10	0.90	33.0	13.12	
Apr. 9	1.00	18.33	11.37	Rabbit 4. Delivered 1.00 p.m., Apr. 9. First milk taken 4.00 p.m. of same day.
" 10	1.60	17.60	11.80	
" 12	2.60	14.40	11.37	
" 16	2.40	14.0	13.12	
" 20	1.90	13.6	13.57	
" 25	2.00	16.0	11.37	
May 1	1.90	13.2	12.23	Rabbit 5. Delivered Apr. 18, a.m. First milk obtained about 4 hrs. later.
Apr. 18	1.1	44.4	21.87	
" 20	1.8	13.4	14.00	
" 25	1.9		15.75	
May 1	1.8	16.6	11.8	

TABLE II.
Analyses of Cat's Milk.

Date of milking.	Lactose.	Fat.	Protein.	Remarks.
1918	per cent	per cent	per cent	
Mar. 25	2.3			Cat 1. Five kittens born Mar. 24, p.m.
" 26	2.5	10.5	6.5	
Apr. 2	4.0	10.6	9.6	
" 10	3.8	10.6	10.9	
" 12	3.8	10.5	15.3	
" 16	3.4	17.3	11.7	
" 20	3.9	8.2	10.5	
May 31	4.0	9.2	10.9	
Apr. 11	2.4	25.5	10.9	Cat 2. Three kittens born during night of Apr. 10-11.
" 12	2.3	10.3	15.3	
" 16	3.9		13.6	
" 20	3.9	10.0	8.3	
" 25	3.8	7.0	6.5	
May 1	3.8	6.2	10.5	
Apr. 9	3.0	18.1	7.87	Cat 3. Six kittens born 5 p.m., Mar. 25.
" 12	3.0	14.3	9.62	
" 16	2.9	12.3	14.00	
" 20	3.9	8.3	9.62	
" 23	3.8	8.6	10.00	

TABLE III.
Analyses of the Milk of Various Animals.

Animal.	Lactose.	Fat.	Protein.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Guinea pig.	3.0	8.3	8.75	Milk taken 24 hrs. after delivery.
Pig.	4.0	3.68	7.93	" " 5 wks. " "
Goat 1.	4.7	2.4	2.14	Pure Saanen (Swiss) goat. Milk taken 8 wks. after kidding.
" 2.	4.9	5.4	2.44	Grade $\frac{3}{4}$ Anglo-Nubian goat. Milk taken 4 $\frac{1}{2}$ mos. after kidding.
" 3.	5.46	4.2	3.32	Grade Anglo-Nubian goat, 50 per cent pure. Milk taken 2 $\frac{1}{2}$ mos. after kidding.
" 4.	5.2	3.7	3.72	Common American goat. Milk taken 3 mos. after kidding.
Sheep.	5.4	2.6	5.00	Hampshire. 4 yrs. old. Milk taken 2 $\frac{1}{2}$ mos. after delivery.
Dog.	2.6	10.0	10.60	Russian wolf hound. Pups 3 days old.
Mare 1.	8.5	0.9	2.40	
" 2.	7.7	1.0	2.70	Before being nursed by foal.
" 2.	8.0	1.6	2.70	After " " " "
" 3.	7.4	0.1	1.72	Average of results on 3 samples. Foal 3 $\frac{1}{2}$ mos. old.
Rabbit.	1.8	12.1	11.4	Average of results on 19 samples of milk from 6 rabbits.
Cat.	3.4	10.9	11.1	Average of results of 26 samples of milk from 4 cats.
Cow.	4.54			Average of results on milk from 21 cows.
Woman.	7.06		1.39	Average of results on milk from 87 normal women.

METHODS FOR THE QUANTITATIVE DETERMINATION OF THE NON-PROTEIN NITROGENOUS CON- STITUENTS OF MILK.

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Boston.)

(Received for publication, January 25, 1919.)

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About a year ago work was commenced on a study of the non-protein fraction of milk. When this investigation was planned it was believed that the methods now in use for the determination of the non-protein constituents of blood could be used with advantage for the analysis of milk. Preliminary experiments soon proved, however, that our supposition was incorrect. The relatively large amounts of lactose and of fat present in milk, and the difference in the chemical properties of the proteins of milk as compared with those of blood, have made it necessary for us to spend a considerable amount of time on working out analytical methods to fit our needs. The methods described below have been used by us in the examination of a considerable number of samples of cow's and human milk and with this material have given good results.

Total Non-Protein Nitrogen.

A necessary preliminary to the determination of non-protein nitrogen in milk is a procedure for removing not only the protein, but also lactose and fat. The necessity of complete removal of protein is obvious, and, while it might be possible to devise some

method of oxidation which would cope successfully with the problem of oxidizing the large quantities of fat and sugar present in milk, the easiest solution of the question lies in preliminary removal of these substances.

Several procedures have been proposed for this determination. The older investigators use alcohol as a protein precipitant. Ritthausen¹ and later Munk² have recommended cupric hydroxide, Siebelein³ has made extensive use of tannic acid, while Simon⁴ has obtained good results with *m*-phosphoric and trichloroacetic acids.

In the procedure described below the desired result is obtained by two precipitations; in the first protein and fat are removed by means of copper sulfate and heat, while in the second the lactose is disposed of by means of cupric hydroxide. We have found that the method of Salkowski⁵ for the precipitation of glucose by alkaline copper solutions may also be applied to lactose, but have found the use of calcium hydroxide as recommended by Van Slyke⁶ superior for our purpose to the sodium hydroxide used by Salkowski, as the excess of calcium hydroxide remaining in solution may be easily and quantitatively precipitated as calcium oxalate. Ammonium salts, urea, creatine, and creatinine may be carried through the above precipitations without loss. Uric acid is completely precipitated so that our results do not include the nitrogen of this substance. As the uric acid nitrogen of milk is but a small fraction of the total non-protein nitrogen, the error due to its loss is small. For some time we found it impossible to effect even an approximately complete recovery of amino-acids; in some cases as much as 65 per cent of the amino nitrogen added (in the form of alanine or leucine) was lost during the process. The property possessed by amino-acids to precipitate in the form of insoluble copper compounds, when these bodies are treated with alkaline cupric hydroxide, has long been known and has of recent years been made use of by Kober and

¹ Ritthausen, H., *J. prakt. Chem.*, 1877, xv, 329.

² Munk, I., *Virchows Arch. path. Anat.*, 1893, cxxiv, 501.

³ Siebelein, J., *Z. physiol. Chem.*, 1889, xiii, 157.

⁴ Simon, G., *Z. physiol. Chem.*, 1901, xxxiii, 466.

⁵ Salkowski, E., *Z. physiol. Chem.*, 1879, iii, 79.

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxii, 455.

Sugiura⁷ for the quantitative determination of amino nitrogen. We have found it possible to prevent the formation of these insoluble amino-copper compounds, and incidentally to obtain complete recovery of amino-acids carried through the three precipitations, by the addition of a small amount of ammonia-free formaldehyde, after the first precipitation and before the addition of lime.

The method as finally worked out for use with cow's milk is as follows: 10 cc. of milk are pipetted into a 100 cc. volumetric flask, and to this are added approximately 50 cc. of water and 20 cc. of 10 per cent copper sulfate solution to which has been added a sufficient amount of sulfuric acid to make a 0.005 N solution. The flask is shaken and placed in a boiling water bath for 20 minutes. It is then removed from the water bath, cooled, made up to volume with distilled water, and the contents are filtered through a dry filter paper. 75 cc. of this filtrate are transferred to a 100 cc. volumetric flask and treated with 1 cc. of 30 per cent formaldehyde and 20 cc. of a 10 per cent suspension of calcium oxide, and after standing at room temperature for 30 minutes the solution is made up to volume and filtered through a dry filter paper. The prescribed time given for the precipitation should not be curtailed, as, if this is done, complete precipitation of lactose will not be secured. If, on the other hand, the time of standing is unduly prolonged to an hour or more, decomposition of the formaldehyde begins with slow evolution of gas and consequent foaming and loss of material.

The filtrate should be clear and colorless, free from lactose, and practically free from all but minute traces of copper. This filtrate is then treated with 0.80 gm. of a mixture consisting of five parts of powdered oxalic acid and two parts of powdered potassium oxalate and centrifuged to remove the precipitated calcium oxalate.

While the amount of precipitant recommended removes in the great majority of cases all the calcium, it is well, after the tubes are removed from the centrifuge, to add a small crystal of oxalate to make sure that the precipitation is complete. 20 cc. of this filtrate (which is equivalent to 1.5 cc. of milk) are pipetted into a

⁷ Kober, A., and Sugiura, K., *J. Am. Chem. Soc.*, 1913, xxxv, 1546.

20 by 200 mm. pyrex glass test-tube; a glass bead or fragment of quartz, and 1 cc. of a digestion mixture prepared by mixing 100 cc. of 85 per cent phosphoric acid, 300 cc. of concentrated sulfuric acid, and 15 cc. of 10 per cent copper sulfate solution are added, and the mixture is evaporated almost to dryness either with a direct flame or in a bath of saturated calcium chloride solution. When practically all the water has been driven off, the tube is heated by means of a microburner for 1 minute after the contents become colorless. During the final period of heating the mouth of the tube should be kept covered by means of a watch-glass or an inverted 25 cc. Erlenmeyer flask. The total time of heating after the evaporation has been completed should not consume more than 3 minutes. As soon as the tube and its contents have cooled, water is added and the ammonia determined either colorimetrically by direct Nesslerization according to the method for the determination of non-protein nitrogen in blood described by Folin and Denis,⁸ or by titration with 0.02 N sodium hydroxide and methyl red after aeration according to the method of Folin and Farmer,⁹ or distillation according to the procedure of Bock and Benedict.¹⁰ In practically all of our work we have used the colorimetric method, but we have had sufficient experience with the other procedures to know that good results are obtainable.

While the procedure outlined above gives excellent results with cow's milk, it requires considerable modification as regards the first precipitation before it can be used satisfactorily with human milk. When human milk is to be used, the precipitation of protein and fat is conducted as follows:

To 10 cc. of milk contained in a 100 cc. volumetric flask are added 20 cc. of 10 per cent copper sulfate solution (which should not contain added sulfuric acid as called for in the directions for use with cow's milk), about 30 to 50 cc. of water, and 1.5 cc. of 10 per cent disodium phosphate solution. The flask is heated for 20 to 30 minutes in a boiling water bath, then cooled, made up to volume with distilled water, and filtered through a dry filter paper. The remainder of the process is carried out as described for cow's milk.

⁸ Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

⁹ Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.

¹⁰ Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.

In Table I are given a few of the results obtained when known amounts of pure solutions of some of the constituents of the non-protein fraction of milk were carried through the process outlined above.

TABLE I.

Results on Recovery of Several Constituents of Non-Protein Fraction of Milk.

Substance added.	Quantity added.	Quantity recovered.
	mg.	mg.
Urea.....	2.00	1.90
“	3.00	2.93
“	5.00	5.10
Alanine N.....	0.50	0.54
“ “	1.00	0.98
“ “	2.00	1.98
Leucine N.....	0.50	0.50
“ “	2.00	2.00
Creatinine.....	0.15	0.16
“	1.00	0.98
Creatine.....	0.50	0.50
“	2.00	1.97
Uric acid.....	0.20	0
“ “	1.00	0
“ “	2.00	0

Urea.

Urea may be determined by Marshall's urease¹¹ method which is well suited for use with milk. We have found it more convenient for our purpose to determine the ammonia produced from the urea colorimetrically instead of by titration as recommended by Marshall. The procedure as used by us contains no new features and the description given below is inserted here merely for completeness.

3 cc. of human, or 5 cc. of cow's milk, are allowed to stand for 30 minutes in contact with 2 cc. of an extract of Jack bean. 1 gm. of solid potassium carbonate and one to two drops of kerosine are then added, and the mixture is aerated for 15 minutes. The ammonia is collected in a 100 cc. volumetric flask containing 2 cc. of 0.1 N hydrochloric acid and 25 cc. of water. At the expiration

¹¹ Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.

of the period of aeration the contents of the flask are made up with distilled water to a volume of 60 to 70 cc. and there are added 15 cc. of Nessler's solution; distilled water is then added to the mark and the solution read in a colorimeter against a suitable standard. For normal milk, the most convenient standard is one containing 0.5 mg. of ammonia nitrogen in a volume of 100 cc.

Creatinine and Creatine.

The only quantitative method for the determination of creatinine and creatine in milk is that of Folin.¹² This method (as regards its application to blood) has recently received severe criticism from several sources.¹³ When an attempt was made to use this procedure it was found that the strictures attached to the method for blood analysis also apply in the case of milk examination; in fact, the rapid increase in color noted when blood filtrates are used, an increase which, as has been pointed out, is not in proportion to changes taking place in the creatinine standard, is even more pronounced in milk than in blood.

Recourse was therefore had to a procedure which has in our hands given good results with blood.¹⁴ This modification consists in the use of *m*-phosphoric acid as a protein precipitant instead of picric acid as recommended in the original Folin method. While this *m*-phosphoric acid filtrate gave good results with blood, it was found that in the case of milk the results were no better than those obtained by the picric acid precipitation. It was possible to prove experimentally that the rapid increase in color, obtained when alkali and picric acid are added to deproteinized milk filtrates (whether the protein precipitant consisted of *m*-phosphoric, trichloroacetic, or picric acid) is largely if not entirely due to the relatively high lactose content of these filtrates; the problem of securing a method for the relatively accurate determination of creatinine and creatine in milk may therefore be solved in one of two ways. In the first we have added to the

¹² Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

¹³ Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413. Hunter, A., and Campbell, W. R., *ibid.*, 1917, xxxii, 195. Greenwald, I., and McGuire, G., *ibid.*, 1918, xxxiv, 103.

¹⁴ Denis, W., *J. Biol. Chem.*, 1918, xxxv, 513.

standard creatinine solution an amount of lactose equivalent to that contained in the filtrate, as a result of which unknown and standard darken at the same rate of speed. The procedure is as follows:

10 cc. of milk contained in a 50 cc. volumetric flask are treated with an equal volume of water and 3 cc. of a freshly prepared 20 per cent solution of *m*-phosphoric acid, and after standing for an hour the flask is filled to the mark with distilled water and the contents are mixed and poured on a dry filter. For the determination of preformed creatinine, 10 cc. of this filtrate are transferred to a 25 cc. volumetric flask containing 10 cc. of picric acid solution (1.2 per cent) and 1.5 cc. of 10 per cent sodium hydroxide. After standing at room temperature for 10 minutes the solution is diluted to volume, placed in the cup of a colorimeter, and read against a standard which has been standing for the same length of time. For normal cow's milk this standard consists of 10 cc. of a 0.9 per cent solution of lactose, 0.025 mg. of creatinine, 10 cc. of picric acid solution, and 1.0 cc. of 10 per cent sodium hydroxide made up to a final volume of 25 cc. For determinations on human milk a 1.4 per cent solution of lactose is substituted for the 0.9 per cent solution used in the case of cow's milk.

For the determination of total creatinine (creatine + creatinine) 10 cc. of the *m*-phosphoric acid filtrate are autoclaved at 120°C. for 30 minutes. After cooling, the solution is treated with picric acid and sodium hydroxide as described above, and after standing for 10 minutes is diluted to a volume of 50 cc. The standard for this determination contains 0.05 mg. of creatinine in a final volume of 50 cc. Lactose solution, etc., is added as described above. By the use of this method on a considerable number of samples of milk, both cow's and human, we obtained values for preformed creatinine of 1.1 to 1.4 mg. of creatinine per 100 cc. of milk, and of total creatinine, 3.1 to 4.0 mg. per 100 cc. of milk, values approximately half as great as those obtained by the Folin method. We have therefore attempted to check our results obtained by the above procedure by resorting to an entirely different method for getting rid of the interfering substances. To this end we have sought to obtain a method whereby the lactose and other active substances might be

removed from the reaction without at the same time destroying any creatine or creatinine. We have found such a precipitant in Salkowski's alkaline cupric hydroxide which precipitates lactose, fat, and protein without carrying down any creatine or creatinine.

The precipitation is best accomplished by the use of copper sulfate and calcium hydroxide as recently suggested by Van Slyke. The procedure is as follows: To 10 cc. of milk contained in a 50 cc. volumetric flask add 5 cc. of a 20 per cent solution of copper sulfate and 15 cc. of a 10 per cent suspension of calcium oxide. Add water to the mark, mix, and allow to stand for 30 minutes; centrifuge, and filter the supernatant liquid through a small dry filter paper. The filtrate should be absolutely clear and colorless and free from lactose and all but minute traces of protein.

To determine preformed creatinine transfer 10 cc. to a 25 cc. volumetric flask, add two drops of normal hydrochloric acid, 10 cc. of saturated picric acid, and 1 cc. of 10 per cent sodium hydroxide solution; let stand 10 minutes, then make up to the mark and read against a suitable standard. For normal milk a standard containing 0.025 mg. of creatinine in 10 cc. of distilled water, 10 cc. of picric acid, and 1 cc. of 10 per cent sodium hydroxide made up to 25 cc. volume is most convenient. For pathological material more concentrated standards are sometimes necessary. For the determination of total creatinine 10 cc. of filtrate are placed in a 50 cc. volumetric flask, treated with two drops of normal hydrochloric acid and 10 cc. of picric acid, and autoclaved for 30 minutes at 120°C. When cool, the mixture is treated with 1 cc. of 10 per cent sodium hydroxide and after standing for 30 minutes is made up to volume and read in a colorimeter against a suitable standard. For normal milk, the most convenient standard is one made to contain 0.05 mg. of creatinine, 10 cc. of picric acid, and 1 cc. of 10 per cent sodium hydroxide in 50 cc.

In the above determinations the addition of sodium hydroxide causes a precipitation of calcium hydroxide which must be centrifuged off before an attempt is made to use the solution in the colorimeter.

The values obtained on normal milk by this method are essentially the same as those obtained when the lactose standard is used and, as we believe it to be theoretically more sound in principle than the former procedure, we have in the latter part of this work adopted it exclusively.

Amino Nitrogen.

The amino nitrogen may be determined by means of Van Slyke's¹⁵ nitrous acid method. For work with milk the micro-apparatus should be used. At first an attempt was made to use 95 per cent ethyl alcohol as a protein precipitant as recommended by Van Slyke and Meyer¹⁶ in their work on blood. While with sufficient care the Van Slyke-Meyer technique was found to give quantitative results in recovering amino-acids added to milk, a short trial convinced us that it was not a practical method for use with this fluid, as so much of the fat present in milk is extracted by the alcohol and later deposited in the deaminizing bulb, and to some extent in the burette, that it was found difficult, if not impossible, to make accurate readings of the latter; an added disadvantage is the fact that the presence of such large quantities of fat makes it necessary to clean the instrument thoroughly after each determination.

Precipitation of the protein by acetic acid followed by trichloroacetic acid as recommended for blood by Bock¹⁷ was also attempted, but was found to be so time-consuming on account of slow filtration that it was abandoned.

After a trial of various protein precipitants we have decided on the use of copper acetate and acetic acid as being most satisfactory for this determination, since by this means it is possible to obtain clear, rapidly filtering filtrates without loss of amino nitrogen. The method as finally worked out for cow's milk is as follows:

20 cc. of milk are pipetted into a 200 cc. volumetric flask, and there are then added 40 cc. of 0.01 N acetic acid, 10 cc. of 5 per cent copper acetate, and 50 to 60 cc. of distilled water. The flask is placed in a boiling water bath for 20 to 30 minutes and at the end of this period of heating, 1 cc. of 15 per cent potassium oxalate solution is added and the mixture cooled, made to volume, and filtered through a dry filter paper. To the filtrate there are now added 0.5 gm. of powdered potassium oxalate, and after shaking the flask for 1 or 2 minutes the precipitate may be re-

¹⁵ Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

¹⁶ Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

¹⁷ Bock, J. C., *J. Biol. Chem.*, 1916-17, xxviii, 357.

moved by centrifuging, leaving a perfectly clear supernatant liquid.

The precipitate may, if desired, be removed by filtration instead of by centrifugation, but in this case the liquid must be allowed to stand for 1 hour as otherwise it is difficult, if not impossible to obtain a clear filtrate.

The addition of potassium oxalate is made in order to remove calcium salts, and while the two filtrations as outlined above may seem unnecessary and cumbersome, it has been found impossible to obtain a filtrate which would remain clear on boiling when all the oxalate was added in the first precipitation; while if the second addition of oxalate is omitted so much solid material precipitates out when the filtrate is evaporated to small volume in preparation for the amino nitrogen determination that it is practically impossible to effect a quantitative transfer to the deaminizing bulb.

For the amino nitrogen determination 50 cc. of the final filtrate are evaporated to a volume of 1 to 2 cc. and the determinations are carried out by means of Van Slyke's micro-apparatus.

For human milk the method of precipitation described above gives poor results, so that with this fluid the following method should be used.

20 cc. of breast milk contained in a 200 cc. volumetric flask are treated with 10 cc. of 5 per cent copper acetate solution, 1 cc. of a 10 per cent solution of disodium phosphate, and 60 to 80 cc. of distilled water. The mixture is heated in a boiling water bath for 20 to 30 minutes, then cooled, made up to volume with distilled water, and the contents are mixed and poured on a dry filter. 50 cc. of the filtrate are evaporated to a volume of about 2 cc. and used for the determination of amino nitrogen. The addition of two to three drops of glacial acetic acid facilitates transfer to the deaminizing bulb.

The advisability of evaporating solutions of amino-acids in open vessels in the presence of relatively large quantities of copper and lactose may well be questioned. The following results indicate, however, that under the experimental conditions described above no destruction of amino nitrogen can be demonstrated during this procedure. These results were obtained on an amino-acid mixture prepared by hydrolyzing Kahlbaum's pure casein with

20 per cent sulfuric acid for 40 hours. The greater part of the sulfuric acid was then precipitated by means of barium hydroxide and the solution finally brought to a faintly acid reaction by means of sodium hydroxide. Amino nitrogen determinations were then made on 1 cc. of the solution (a) without the addition of any other substance, (b) with the addition of 0.2 gm. of copper sulfate to 1 cc. of the solution, (c) with the addition of 0.2 gm. of copper sulfate and 0.1 gm. of lactose to 1 cc. of the solution. In (b) and (c) the mixture was made up to a volume of approximately 50 cc. and evaporated on the sand bath to a volume of 1 to 3 cc. under exactly the same experimental conditions as were used for the determination of amino nitrogen in milk. The results are given in Table II.

TABLE II.

Amino N per 100 cc. of solution before treatment.	Amino N per 100 cc. of solution after heating with CuSO_4 .	Amino N per 100 cc. of solution after heating with CuSO_4 and lactose.
mg.	mg.	mg.
30.34	31.48	32.00
31.48	31.48	32.00
31.48	32.00	
32.00		

TABLE III.

Comparisons of Results Obtained on Cow's Milk by Alcohol and by Copper Precipitations.

No. of milk.	Amino N per 100 cc. milk.	
	Alcohol precipitation.	Copper precipitation.
	mg.	mg.
4	4.86	4.86
3	4.52	4.62
10	5.74	5.78

In Table III are presented a few results in which amino nitrogen was determined in cow's milk after alcohol precipitation by the technique of Van Slyke and Meyer and by the copper precipitation described above. The results obtained by the two methods are almost identical.

Uric Acid.

Uric acid determinations in blood have almost invariably been made with Salkowski's ammoniacal silver-magnesium reagent, preceded by precipitation with acetic acid and evaporation to small volume. Such a procedure is absolutely unsuited for use with milk on account of the large quantities of lactose present, which, when the milk filtrates are evaporated to small volume, leaves a sticky mass producing strong reduction of the ammoniacal silver reagent.

For our purpose the method of precipitation of uric acid with alkaline zinc acetate solution recommended by Morris¹⁸ has proved excellent, as by this method it is possible to remove quantitatively small amounts of uric acid from relatively large volumes of lactose solution. Recently Curtman and Lehrman¹⁹ have suggested the use of nickel acetate in place of zinc acetate as recommended by Morris. In a trial of nickel as a uric acid precipitant we have obtained excellent results, but as we have been unable to find that for our purpose this reagent possesses any advantage over zinc acetate we have continued our use of the latter.

The method as finally worked out is as follows: 20 cc. of milk contained in a 100 cc. volumetric flask are treated with 40 cc. of 0.01 N acetic acid and 1 cc. of 10 per cent zinc acetate solution; the mixture is heated in a boiling water bath for 15 to 20 minutes and is then cooled, made up to volume, and filtered through a dry filter paper. The filtration should be rapid and the filtrate absolutely clear. 75 cc. of filtrate are treated with 3 cc. of a 10 per cent solution of zinc acetate and 4 cc. of 20 per cent sodium carbonate solution, allowed to stand at room temperature for 10 minutes, and then centrifuged. The gelatinous precipitate of zinc carbonate settles rapidly and completely, is washed once with cold water, and the tubes are then centrifuged a second time. After pouring off the wash water as completely as possible the residue is treated with 2 cc. of 50 per cent acetic acid, then transferred by means of about 25 to 35 cc. of water to an Erlenmeyer flask; the contents of the flask are heated to boiling, and the

¹⁸ Morris, J. L., *J. Biol. Chem.*, 1916, xxv, 205.

¹⁹ Curtman, L. J., and Lehrman, A., *J. Biol. Chem.*, 1918, xxxvi, 157.

zinc is precipitated with hydrogen sulfide. When precipitation is complete, the liquid is filtered, the zinc sulfide precipitate washed twice with hot water, the filtrate and washings are boiled rapidly until entirely free from hydrogen sulfide, and the volume is re-

TABLE IV.

Results on Recovery of Non-Protein Bodies Added to Cow's Milk.

Substance added.	Mg. per 100 cc. milk.			
	Amount present.	Amount added.	Amount recovered.	Theory.
Urea N.....	10.0	5	14.8	15.0
" ".....	10.0	10	20.2	20.0
" ".....	10.0	10	19.8	20.0
Amino N.....	7.02	2	8.98	9.02
" ".....	7.02	4	11.42	11.02
" ".....	7.02	6	12.91	13.02
Creatinine.....	1.1	1	2.0	2.1
" ".....	1.1	2	3.0	3.1
Creatine.....	2.2	1	3.1	3.2
" ".....	2.2	2	4.1	3.9
Uric acid.....	1.5	1	2.2	2.5
" ".....	1.5	3	4.3	4.5
" ".....	1.5	5	6.4	6.5

TABLE V.

Non-Protein Nitrogenous Constituents of Milk.

Source of material.	Mg. per 100 cc. milk.					
	Total non-protein N.	Urea N.	Amino N.	Preformed creatinine.	Creatine.	Uric acid.
Cow, Dairy I.....	21.9	10.0	4.03	1.35	2.30	1.4
" " II.....	21.0	9.0	4.19	1.40	2.40	1.6
" " III.....	26.0	9.0	4.50	1.5	2.40	1.6
Mother I.....	26.0	12.0	4.58	1.1	3.9	2.2
" II.....	37.0	12.1	8.46	1.6	3.2	2.7
" III.....	36.0	13.8	8.58	1.8	3.4	2.7

duced to approximately 15 cc. After cooling, 1 cc. of phosphotungstic acid reagent²⁰ and 10 cc. of 20 per cent sodium carbonate solution are added and the volume is made up to 25 or 50 cc. ac-

²⁰ Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

according to the degree of color. Readings are then made in a colorimeter against a suitable standard uric acid solution. The standard which is most generally useful is one containing 0.5 mg. of uric acid in a volume of 100 cc.

The following results indicate the completeness with which uric acid can be recovered by the above procedure from relatively large quantities of lactose solution:

Uric acid added.	7 per cent lactose solution.	Uric acid recovered.
<i>mg.</i>	<i>cc.</i>	<i>mg.</i>
0.2	10	0.19
1.0	50	1.0
1.0	100	0.97

In Table IV are presented the results obtained by adding known quantities of urea, amino-acids, creatine, creatinine, and uric acid to cow's milk.

Table V contains the results obtained on a few samples of cow's and human milk. These results are introduced to give an idea of the general level at which the non-protein nitrogenous bodies occur in these fluids.

The cow's milk was purchased in the open market and represents the product sold by three large milk distributors in Boston. The samples of human milk were from three normal women. For the latter specimens we are indebted to Dr. Fritz Talbot.

For some time we have been engaged by the help of the methods described above in the accumulation of data concerning the non-protein constituents of milk. The results of this work will be published shortly.

DETERMINATION OF THE DISTRIBUTION OF NITROGEN IN CERTAIN SEEDS.*

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Recent progress in the chemistry of protein nutrition has demonstrated the importance of the rôle played by the amino-acids and sufficient evidence is at hand to show that protein nutrition is in reality amino-acid nutrition. Therefore for the purposes of human nutrition and of animal husbandry an exact knowledge of the amino-acid content of foodstuffs is essential. Methods of protein analysis which furnish accurate results expeditiously are therefore of the utmost importance. The Van Slyke method when applied to isolated and purified proteins partially meets this need but, obviously, the method would be of still greater value if it could be applied to the direct analysis of seeds without previous isolation of the proteins. In the investigation herein presented this was attempted with the hope of obtaining at least approximate results.

It is evident, however, that the possible sources of error in such an application of the Van Slyke method are many. Gortner and Blish¹ have shown that when tryptophane or a protein containing this amino-acid is hydrolyzed with hydrochloric acid in the presence of dextrose, the humin nitrogen is greatly increased. Grindley and Slater² regard the presence of carbohydrates to be a source of error in the hydrolysis of seed material for the purpose of determining its amino-acid content. They intimate, moreover,

* A brief notice of this work was published in *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 192.

¹ Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.

² Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

that possibly the presence of glycerol or of glycerides may increase the humin nitrogen.

In the consideration of sources of error in the determination of the amino-acid content of seeds, it seems worth while to take into account the presence in the seeds of forms of nitrogen other than protein, such as amines and free amino-acids, and, important in this sort of analysis, the nucleic acid which is a part of the seed embryo. Information concerning the plant nucleic acids is limited. That of yeast and that of the wheat embryo have been thoroughly investigated by Osborne³ and by Levene,⁴ who believe that the two are practically identical, and it is probable that all the plant nucleic acids are closely related or have a very similar structure.

TABLE I.

Nitrogen Distribution in Nucleic Acid of Yeast as Determined by the Van Slyke Method.

Sample, 3 gm.	Total N.	Amide N.	Humin N with lime.	Humin N, bases.	Total N, bases.	Amino N, bases.	Arginine N.	Total N, filtrate.	Amino N, fil- trate.
Gm.	0.4752	0.1140	0.05166	0.1074	0.1324	0.024	0.0714	0.0602	0.017
Per cent of total (uncor- rected).		24.00	10.87	22.09			15.03	12.66	

For the purpose of determining the behavior of plant nucleic acid in the Van Slyke analysis, yeast nucleic acid, which was free from material giving the biuret reaction and did not respond to qualitative tests for ammonium acetate, was hydrolyzed 25 hours with 20 per cent hydrochloric acid and subjected to the usual procedure.

Examination of the results given in Table I shows that a high percentage of humin nitrogen was precipitated with the bases, which may be explained by the fact that it is impossible to decompose completely the phosphotungstate precipitate of the bases with ether-amyl-alcohol mixture and dilute hydrochloric acid as recommended by Van Slyke.⁵ Although yeast nucleic acid does

³ Osborne, T. B., and Harris, I. F., *Z. physiol. Chem.*, 1902, xxxvi, 85.

⁴ Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1910, xliii, 3164.

⁵ Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

not contain arginine, the analysis indicates the presence of 15 per cent of arginine nitrogen. This is probably derived from the guanine, adenine, and cytosine of the nucleic acid, since these compounds are decomposed by boiling with concentrated caustic alkali as in the procedure used for the determination of arginine.

Table II gives the results of analyses of cottonseed flour, tomato seed flour, cow peas, jack beans, corn, corn germ, wheat, kafir, and kafirin, the alcohol-soluble protein of kafir.⁶ With the exception of cottonseed flour and tomato seed flour, the seeds and grains were prepared for analysis by fine grinding in a suitable mill. They were used without further drying and without extracting the fats. The cottonseed flour was the well known commercial article. Tomato seed flour is the ground press cake of tomato seeds obtained as a by-product in the manufacture of tomato pulp. The seeds yield, on pressing, from 15 to 20 per cent of oil. Portions of the ground material were weighed, hydrolyzed with 20 per cent hydrochloric acid, and then treated exactly as recommended by Van Slyke.

By comparing the results given in Table II with those of others who have analyzed the same sort of material (Grindley and Slater,² Nollau,⁷ and others), it will be noted that there is a widely varying agreement. The results of Nollau have been criticized by Grindley and Slater, who pointed out that Nollau's procedure was not comparable with theirs. Grindley and Slater followed the same procedure that was used in obtaining the results recorded in Table II, and these results agree fairly well with theirs. Differences noted may be due to the fact that different samples of seeds may vary in composition.

In the analysis of kafir corn and of tomato seed a deficiency in histidine nitrogen was found, calculations yielding minus quantities. The presence of histidine in tomato seed is yet in doubt. The solution of the hexone bases of kafir gives a positive reaction with bromine for this amino-acid, and histidine nitrogen, 1.64 per cent corrected, was found in the kafir prolamine. It seems justifiable then in the case of the whole kafir to assume that most of the small amount of histidine present remained in solution and,

⁶ Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916-17, xxviii, 59.

⁷ Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

TABLE II.
Distribution of Nitrogen in Certain Seeds as Determined by the Van Slyke Method.

Nitrogen in per cent of total nitrogen.												
Weight of sample.	Total N.	Nitrogen in per cent of total nitrogen.										
		Amide N.	Humin N in lime.	Humin N precipitated with bases.	Arginine N.	Histidine N.	Cystine N.	Lysine N.	Amino N in filtrate from bases.	Non-amino N in filtrate from bases.		
gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
4	0.3185	10.00	10.97		19.73	3.87	1.11	6.69	43.50	5.27		
4	0.2622	10.38	12.03		16.32	None found.	1.27	8.39	45.86	5.09		
8	0.3277	9.88	5.34	2.87	17.68	3.64	1.23	5.98	49.12	4.08		
12	0.3969	7.56	6.35	4.11	9.83	6.08	0.79	9.90	45.54	9.40		
12	0.2220	13.94	9.35		7.75	2.46	1.60	2.06	57.86	6.28		
8	0.2648	12.69	14.71		11.04	5.84	None.	5.62	45.07	6.97		
12	0.3333	18.65	6.26	1.25	8.96	1.73	0.87	2.65	48.79	13.37		
12	0.2808	16.34	4.85		7.17	1.32	1.02	1.68	59.01	7.69		
3	0.4716	20.74	0.94	0.07	3.08	1.64	0.55	1.04	65.20	7.29		
Cottonseed flour.....												
Tomato seed (pressed).....												
Cow pea (<i>Vigna sinensis</i>).....												
Jack bean (<i>Canavalia ensiformis</i>).....												
Corn (<i>Zea mais</i>).....												
Corn germ (pressed).....												
Wheat.....												
Kafir corn (<i>Andropogon sorghum</i>).....												
Kafrin.....												

applying the correction for solubility of histidine, 1.32 per cent is recorded.

The figure for cystine nitrogen in kafirin recorded was also obtained by adopting the factor for solubility of the phosphotungstate. The barium sulfate precipitate, though unweighable, was present, and as the reagents used give no precipitate of barium sulfate in blank, it appears safe to adopt Van Slyke's correction of 0.0026 gm. as representing all the cystine nitrogen in solution.

CONCLUSIONS.

It is shown that when yeast nucleic acid is subjected to the Van Slyke procedure, 15 per cent of the total nitrogen of the acid appears in the arginine fraction, although the nucleic acid contains no arginine. This indicates that in the determination of the distribution of the nitrogen in materials containing nucleic acid by the method of Van Slyke, erroneous results may be obtained because some of the purine and pyrimidine nitrogen appears in the arginine fraction.

The nitrogen distribution in certain seeds and seed products was determined by the method of Van Slyke and the results agree in general with those obtained by others who have used the same method. Where there is lack of agreement this might be due to the fact that different samples of seed of the same species may vary in composition.

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REDUCTION OF THE QUANTITY OF HUMIN NITROGEN FORMED IN THE HYDROLYSIS OF THE NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

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(Received for publication, January 28, 1919.)

In view of the importance which individual amino-acids had assumed in the biological evaluation of feedingstuffs, the junior author of this paper, with the assistance of Joseph, Slater, and Eckstein, attempted quantitatively to determine directly the combined amino-acids of feedingstuffs by the application of the Van Slyke method for the determination of the characteristic groups of amino-acids of proteins. In the publications¹ of the results of this work, the following facts have been clearly emphasized:

First, "that the Van Slyke method for the determination of the chemical groups characteristic of the amino acids [of proteins] can be applied directly to the quantitative determination of amino acids of feedingstuffs with at least a fair degree of accuracy."

Second, "that the object of the work . . . is to determine the free and the combined amino acids . . . of feedingstuffs, and not merely the amino acids and the amides resulting from the hydrolysis of the proteins of the feedingstuffs."

Third, "that the high results for humin nitrogen [due to the presence of the carbohydrates in feedingstuffs] constitute a source of error in the direct application of the Van Slyke method to the determination of the free and combined amino acids and amides of feedingstuffs."

Fourth, "that further study was being made to determine to what extent, if any, the so-called non-protein nitrogenous substances affect the quantitative determination of the amino acids of feedingstuffs."

¹ Grindley, H. S., Joseph, W. E., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 1778. Grindley, H. S., and Slater, M. E., *ibid.*, 2762. Grindley, H. S., and Eckstein, H. C., *ibid.*, 1916, xxxviii, 1425. Grindley, H. S., *Proc. Am. Soc. Animal Production*, 1916, 133.

Fifth, "that the Van Slyke method for the determination of the chemical groups characteristic of the amino acids of proteins will prove of much value as applied to the quantitative determination of the free and combined amino acids . . . of feedingstuffs when we become better acquainted with the details of the method, and succeed in adapting it to the specific conditions involved. At any rate, as yet, it is the only method that has been used for the determination of the amino acid content of feedingstuffs, and, since it undoubtedly gives approximately quantitative results, it should, until some better method is developed, be used in gaining knowledge, that will aid us in applying to the economic and nutritive valuation of the common feedingstuffs, the fast accumulating results as to the nutritive value of the amino acids."

Since our last publication the methods for this work have been decidedly improved. In the first place, the interference of some of the non-protein nitrogenous constituents such as the nitrogenous lipoids, the nitrogenous coloring matters, betaine, stachydrin, amygdalin, most of the alkaloids, and some of the amides has been obviated by completely extracting first with ether and then with cold absolute alcohol.

In the second place, a method has been perfected by which the percentages of the humin nitrogen formed in the hydrolysis of the nitrogenous constituents of feedingstuffs are reduced so that they compare favorably with those obtained for pure proteins. The essential features of this method are as follows: (1) extraction of the feedingstuffs with ether and then with cold absolute alcohol; (2) the conversion, as far as possible, of the insoluble carbohydrates into soluble carbohydrates by boiling the feedingstuffs with 0.1 per cent hydrochloric acid; (3) the separation of the greater part of the soluble proteins before hydrolysis from the soluble carbohydrates by neutralizing the mineral acid and precipitating with alcohol; (4) the hydrolysis of the small portion of the proteins remaining in solution with the soluble sugars by boiling with 5 per cent instead of 20 per cent hydrochloric acid for only a short time.

By these proceedings it is possible to separate the greater part of the carbohydrates from the main portion of the proteins before the latter are hydrolyzed and thus decidedly reduce the quantity of humin formed. Likewise since it is possible to hydrolyze the small amount of soluble proteins remaining in solution with the carbohydrates with dilute hydrochloric acid in a short time, a further reduction of the quantity of humin is obtained.

This method gives the following results for humin nitrogen expressed in per cent of the total nitrogen in the feedingstuff: corn 3.2, wheat 3.4, oats 4.5, and barley 3.9 per cent. The results for the humin nitrogen which were obtained in our earlier analysis of these feeds were as follows: corn 9.8, wheat 9.2, oats 9.9, and barley 8.8. The humin nitrogen content of some of the pure proteins as analyzed by Van Slyke is as follows: hemoglobin 3.6, fibrin 3.2, and lactalbumin 2.3. It is thus clearly evident that the error due to humin nitrogen in the analysis of these feedingstuffs is no greater than it is in the analysis of some of the pure proteins. From results recently obtained, we feel certain that the quantity of humin nitrogen formed in the hydrolysis of the nitrogenous constituents of feedingstuffs can be still further reduced.²

Briefly stated the details of the method used in this work are as follows: Weighed quantities of the feedingstuff are extracted with ether in Soxhlet extractors and then with cold absolute alcohol on Buchner funnels. The residues thus extracted are digested for 15 hours three or four times with 0.1 per cent solution of hydrochloric acid until all the starch has been converted into sugars. The residues insoluble in 0.1 per cent hydrochloric acid are boiled with 20 per cent hydrochloric acid until the proteins which they contain are completely hydrolyzed.

The filtrates from the residues insoluble in 0.1 per cent hydrochloric acid are neutralized with sodium hydroxide, then faintly acidified with acetic acid, allowed to stand over night, and then filtered. The filtrates from the precipitated proteins are concentrated *in vacuo* to small volumes and precipitated by the addition of five volumes of absolute alcohol. After standing over night, the precipitated proteins are removed by filtration and washed with 83 per cent alcohol.

The filtrates from the proteins precipitated by alcohol are concentrated to small volume and enough concentrated hydrochloric acid is added to make a 5 per cent solution. The solutions are then

² By improvement of the method reported here the humin content of corn has been still further reduced to 2.53 per cent since the manuscript for this paper was prepared.

boiled until hydrolysis is complete.³ The proteins separated above by neutralization and by the addition of alcohol are boiled with 20 per cent hydrochloric acid until hydrolysis is complete.

As a result of the above procedure, there are obtained three different fractions of the proteins of feedingstuffs which are completely hydrolyzed; namely, (1) the proteins insoluble in 0.1 per cent hydrochloric acid; (2) the proteins separated by neutralizing the mineral acid and precipitating with alcohol; (3) the soluble proteins not separated by neutralizing the mineral acid or precipitating with alcohol. Each of the three hydrolyzed solutions is filtered and the insoluble humin substances are repeatedly digested with 0.1 per cent hydrochloric acid and then thoroughly washed with hot water. The nitrogen in these residues is considered to represent the insoluble humin substances.

The filtrates from the insoluble humin were concentrated *in vacuo* to small volume and the amide and soluble melanine nitrogen determined as usual. The remaining parts of the analysis are continued as usual. Only the results for the humin nitrogen are reported here; the complete analysis will be given in a later paper.

³ In all cases it was found that a 5 per cent solution of hydrochloric acid would bring about the complete hydrolysis of the small amount of soluble proteins remaining in these solutions.

EXPERIMENTAL STUDIES ON GROWTH.

X. THE LATE GROWTH AND SENESCENCE OF THE NORMAL WHITE MOUSE, AND THE PROGRESSIVE ALTERATION OF THE NORMAL GROWTH CURVE DUE TO INBREEDING.

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(Received for publication, January 3, 1919.)

Growth and Senescence of Normal White Mice.

In previous articles of this series¹ we have furnished data concerning the normal growth of the white mouse for the first 60 weeks of life, employing for the collection of these data a special technique designed to exclude accidental causes of fluctuation, such as epidemic infections, etc., and, as material for measurement, a large number of animals born in July, 1914, from a stock of animals purchased a few months previously from dealers.

Our observations upon these animals were continued until their death from natural causes, and weighings of every animal in the series were made fortnightly during the whole period of their existence (weekly during the first 30 weeks). We have now to report the complete series of measurements from the 4th week after birth until the termination of the observations by the death of the last surviving animal.² The results of these measurements are enumerated in Tables I and II and illustrated graphically in Figs. 1 and 2, displaying the progressive alterations of the average weight of the animals with increasing age.

¹ Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347. Robertson, T. B., *ibid.*, 363.

² Data concerning growth from birth until the 4th week may be found in the articles cited above, also in Paper IX of this series (Robertson, T. B., and Delprat, M., *J. Biol. Chem.*, 1917, xxxi, 567).

An examination of these curves shows that the average weight of animals of both sexes increases, with a continuously decreasing velocity, to a definite maximum, attained at about 91 weeks of age in the male and at about 94 weeks of age in the female. After attaining this maximum the curve descends rather steeply, the loss of weight being much more rapid than the immediately preceding gain.

This loss of weight is the expression of senescence, and continues without interruption until the decease of the last surviving animal. It will be observed, however, that the curve of growth in both sexes displays marked oscillations towards the end, which are due to the irregularly occurring deaths of animals in which the process of senescence has been most rapid, and which have lost most weight. The survivors therefore represent an earlier or less complete stage of senescence than those which have died, and each group of late deaths is consequently accompanied by a rise in the weight curve of the survivors. Each rise, however, is succeeded by a fall which is even more rapid than the preceding one, indicating that the process of senescence is in reality continuous, and moreover that it proceeds with a regularly increasing velocity depending upon the age rather than upon the weight of the animals. This general feature of the process of senescence is additionally illustrated by the curves accompanying Paper XI of the series.

The average duration of life (110 weeks in the male, 103 weeks in the female) exceeds by a definite interval the period during which the weight of the animals is increasing. The attainment of the average duration of life is therefore preceded by a decided measure of senescence, which must consequently have affected the viability of the majority of the animals and played a leading part in determining the mean duration of life. 78 per cent of the males and 68 per cent of the females survived the attainment of maximum weight, and therefore experienced the senescent loss of weight.

TABLE I.
Normal Males.

Age.	No. weighed.	Weight.	Variabil- ity.	Age.	No. weighed.	Weight.	Variabil- ity.
<i>wks.</i>		<i>gm.</i>	<i>per cent</i>	<i>wks.</i>		<i>gm.</i>	<i>per cent</i>
4	65	12.38	24.4	60	30	29.08	11.8
5	117	12.45	24.6	62	30	29.41	11.6
6	72	15.58	22.0	64	30	29.53	11.4
7	43	18.08	16.9	66	30	29.70	11.4
8	42	19.36	15.9	68	30	29.80	11.1
9	42	20.63	16.5	70	30	29.70	10.9
10	42	21.19	16.7	72	30	29.70	12.2
11	42	21.81	13.3	74	29	29.76	10.9
12	42	22.65	13.7	76	29	29.98	10.0
13	37	23.31	14.2	78	29	30.16	11.5
14	36	23.96	14.1	80	29	29.95	11.1
15	36	24.28	11.9	82	26	30.31	11.3
16	36	24.75	12.7	84	26	29.88	12.3
17	36	25.21	12.4	86	25	30.10	13.0
18	36	25.61	12.1	88	24	30.19	13.0
19	35	25.81	12.1	90	24	30.13	11.3
20	35	26.10	10.8	92	24	30.46	12.0
21	35	26.28	11.2	94	24	30.13	11.1
22	35	26.06	9.0	96	23	29.67	10.1
23	34	26.34	9.8	98	22	29.36	10.0
24	34	26.82	10.1	100	21	29.54	9.6
25	33	27.05	11.0	102	20	29.48	10.7
26	33	26.94	10.8	104	19	29.61	10.4
27	32	26.55	11.3	106	16	29.03	10.7
28	31	27.19	10.9	108	16	28.56	9.6
29	31	27.08	10.9	110	16	28.25	11.0
30	31	27.23	9.5	112	15	28.30	11.3
32	32	27.61	9.6	114	16	28.16	11.3
34	31	27.71	10.5	116	13	28.40	12.6
36	30	27.95	10.1	118	14	28.29	12.8
38	30	28.20	10.5	120	12	27.25	10.9
40	30	28.27	10.4	122	12	27.08	10.6
42	30	28.28	11.2	124	11	27.32	10.2
44	30	28.62	12.0	126	11	26.73	12.3
46	30	28.55	11.7	128	10	27.55	10.6
48	30	28.22	10.8	130	9	28.11	10.8
50	30	28.88	11.1	132	5	28.00	11.2
52	30	28.68	10.7	134	5	28.00	—
54	30	28.67	12.0	136	5	27.50	—
56	30	29.32	12.0	138	2	25.50	—
58	30	29.10	10.9	140	1	26.00	—

TABLE II.
Normal Females.

Age.	No. weighed.	Weight.	Variability.	Age.	No. weighed.	Weight.	Variability.
<i>wks.</i>		<i>gm.</i>	<i>per cent</i>	<i>wks.</i>		<i>gm.</i>	<i>per cent</i>
4	89	10.39	23.4	66	28	26.45	19.2
5	77	11.81	19.4	68	28	26.55	18.3
6	68	14.12	18.9	70	27	26.13	18.7
7	37	16.77	15.0	72	26	26.56	17.7
8	36	17.99	13.9	74	25	26.76	16.9
9	36	18.78	13.4	76	25	27.24	17.7
10	36	19.38	14.3	78	23	27.39	17.0
11	36	20.04	12.6	80	22	27.64	17.4
12	36	20.31	12.6	82	22	27.39	17.0
13	36	21.04	13.0	84	22	27.75	16.4
14	36	21.21	13.2	86	22	27.89	17.6
15	36	21.78	13.7	88	22	27.77	18.7
16	36	22.14	12.6	90	22	27.82	16.8
17	36	22.29	11.9	92	21	27.98	18.4
18	36	22.22	11.9	94	20	27.53	18.0
19	36	22.60	12.0	96	20	28.03	17.8
20	36	22.60	11.2	98	19	27.89	20.9
21	36	23.06	12.3	100	19	27.42	22.2
22	36	23.32	12.5	102	17	27.62	20.7
23	35	23.51	12.0	104	16	27.06	19.1
24	36	23.68	11.3	106	15	26.57	18.6
25	35	23.79	11.4	108	11	26.82	18.2
26	36	24.04	11.6	110	10	26.30	17.7
27	35	24.00	11.8	112	10	26.70	16.0
28	36	23.58	10.3	114	7	24.43	10.5
29	35	23.84	12.2	116	7	24.57	9.7
30	36	23.92	12.4	118	7	24.29	10.0
32	36	24.18	11.5	120	7	24.14	10.0
34	36	24.18	12.2	122	7	24.93	11.0
36	36	24.65	11.2	124	7	24.93	9.5
38	36	24.80	12.3	126	6	24.83	9.8
40	35	25.03	11.9	128	5	25.80	7.8
42	35	25.07	13.1	130	5	25.80	8.1
44	34	25.52	15.3	132	5	25.10	6.6
46	33	25.68	14.6	134	5	25.50	7.8
48	32	25.45	14.6	136	4	24.00	—
50	32	25.50	14.0	138	4	24.50	—
52	29	25.76	15.3	140	4	24.63	—
54	29	25.78	15.8	142	4	24.88	—
56	29	26.00	16.5	144	3	24.00	—
58	29	26.26	16.7	146	3	24.50	—
60	29	26.12	17.8	148	2	24.00	—
62	29	26.40	17.7	150	2	24.00	—
64	29	26.64	17.8				

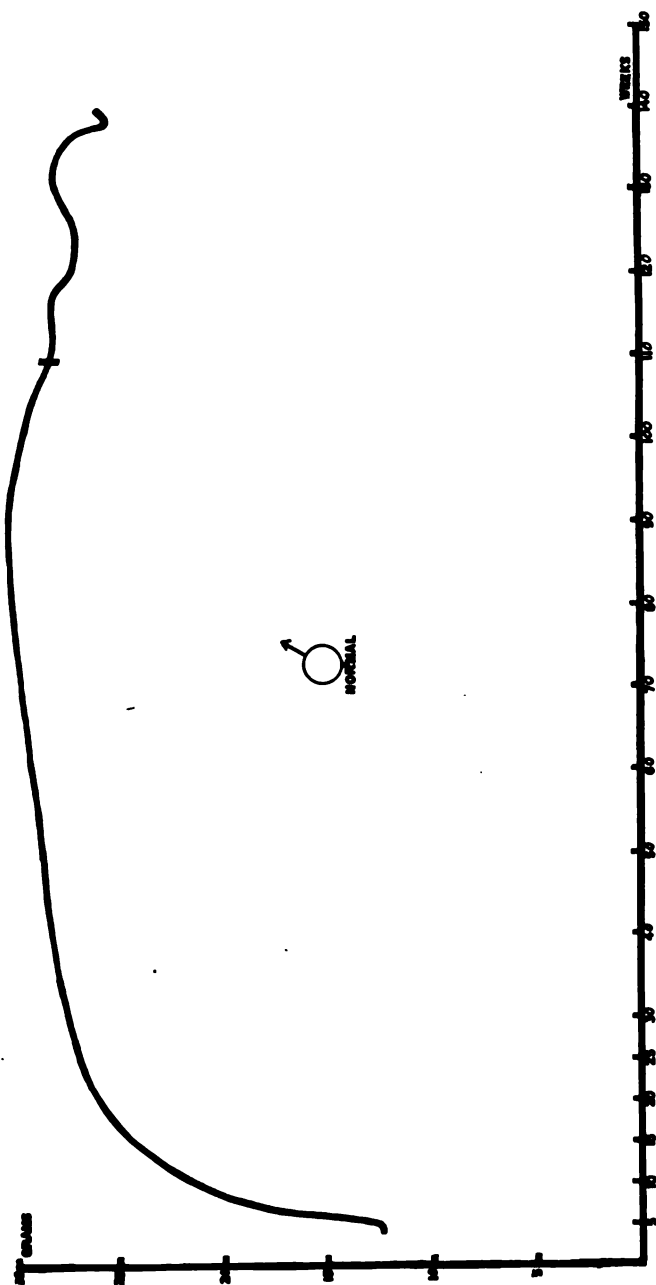


FIG. 1. Growth curve of normal male white mice from 4 weeks until death of the last surviving animal. The vertical cross mark indicates average duration of life.

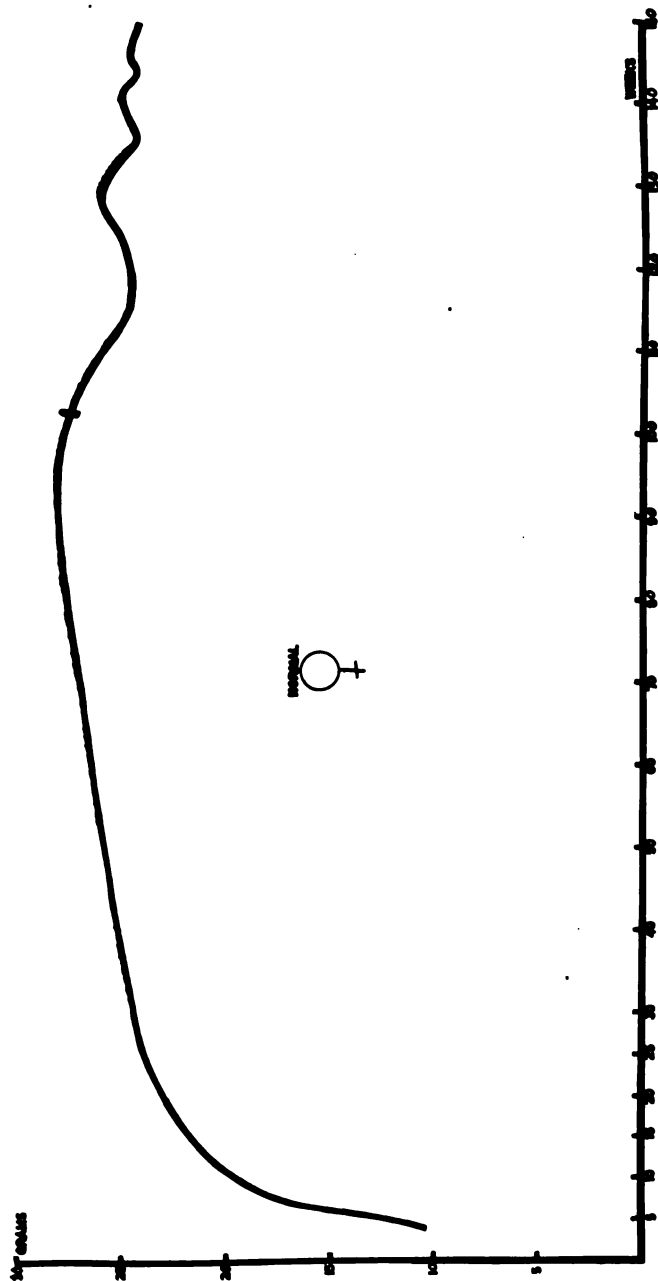


Fig. 2. Growth curve of normal female white mice from 4 weeks until death of the last surviving animal. The vertical cross mark indicates average duration of life.

Progressive Alteration of Normal Growth Curve Due to Inbreeding of Mixed Stock.

Our original stock of animals was obtained from animal dealers, and therefore consisted in all probability of a variety of different strains. We have consistently adopted a plan of rotation whereby it is assured that no female is ever impregnated twice in succession by the same male. In this way thorough mixture of the strains has no doubt been brought about in the course of time, and close inbreeding has been avoided. We have found, however, that successive batches of animals thus obtained display definite and progressive changes in the curve of growth.

Since the changes in question are quantitative, and do not exceed the individual differences which may frequently be observed between the weights of any single pair of animals selected at random, the only method of attaining any degree of certainty as to the reality of the differences is to determine the ratio of the observed average differences between successive batches of normals to the probable error of the experimental estimate of the differences themselves; that is, to $\sqrt{E_1^2 + E_2^2}$ where E_1 and E_2 are the probable errors of the separate estimates of average weight.³

The chance of any given, observed deviation being accidental may be estimated from Table III, computed from a table of areas of the normal frequency curve.⁴ Thus if the ratio of an observed deviation from the standard chosen to the probable error of its estimation is 3.0, then there is one chance that it is accidental to 22 that it is not accidental but systematic in origin; i.e., that it originates in a definite and not fortuitous difference between the two groups of animals investigated.

In Tables IV to VII and Figs. 3 and 4 are given the comparative growth of three successive batches of normal males, born, respectively, in July 1914, November 1914, and July 1916, and of three successive batches of normal females born, respectively, in July 1914, July 1916, and October 1917. The curves reveal a

³ Davenport, C. B., *Statistical methods with special reference to biological variation*, New York, 2nd edition, 1904, 15.

⁴ Davenport, C. B.,³ p. 119.

regularly diminishing velocity and absolute magnitude of growth in each succeeding batch of animals.

The absolute difference between the average weights of July and November 1914 males, averaging from 1.0 to 1.5 gm., is only slightly less than that between the males of November 1914 and July 1916 (1.5 to 2.0 gm.), although a period of 20 months elapsed between the birth of the two latter groups and of less than 5 months between the birth of the two former groups. Nearly as much change in the stock occurred, therefore, in the first 5 months of inbreeding as in the succeeding 20 months.

Owing to the much smaller variability of the animals comprising the later batches, however, the observed deviations only average from two to three times the experimental error for the 1914 batches, while they average between four and six times the experimental error in the case of the November 1914 and July 1916 batches of animals. In both sexes diminution of the variability of the animals, as in other instances previously noted,⁵ is associated with diminished rapidity of growth.

The effects are much smaller in the females, the differences between the 1917 and 1916 batches lying almost entirely within the experimental error of the estimations.

It would appear, therefore, that the animals are approaching, if they have not already reached, a stable type, but that this requires a considerable period of time notwithstanding the deliberate effort made to secure thorough mixing of the strains. From these facts it will be evident that no growth standard, no matter upon how many observations it may be established, is applicable to animals derived from a different strain or stock. Each stock must provide its own standard norm and this norm must be re-established at frequent intervals.

⁵ Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 372; *Am. J. Physiol.*, 1916, xli, 535, 547. Thompson, D. W., *Growth and form*, Cambridge, 1917.

TABLE III.

Ratio of deviation from normal to probable error of estimate.	Chance that observed deviation is accidental.		Ratio of deviation from normal to probable error of estimate.	Chance that observed deviation is accidental.	
0.1	18 to	1	2.8	1 to	16
0.2	7.5 "	1	2.9	1 "	19
0.3	5.2 "	1	3.0	1 "	22
0.4	3.7 "	1	3.1	1 "	26
0.5	2.8 "	1	3.2	1 "	31
0.6	2.2 "	1	3.3	1 "	37
0.7	1.7 "	1	3.4	1 "	45
0.8	1.4 "	1	3.5	1 "	54
0.9	1.2 "	1	3.6	1 "	65
1.0	1 "	1	3.7	1 "	79
1.1	1 "	1.2	3.8	1 "	95
1.2	1 "	1.4	3.9	1 "	116
1.3	1 "	1.6	4.0	1 "	143
1.4	1 "	1.9	4.1	1 "	172
1.5	1 "	2.2	4.2	1 "	214
1.6	1 "	2.5	4.3	1 "	266
1.7	1 "	2.9	4.4	1 "	335
1.8	1 "	3.4	4.5	1 "	415
1.9	1 "	4.0	4.6	1 "	514
2.0	1 "	4.6	4.7	1 "	657
2.1	1 "	5.4	4.8	1 "	832
2.2	1 "	6.3	4.9	1 "	1,041
2.3	1 "	7.3	5.0	1 "	1,310
2.4	1 "	8.5	6.0	1 "	19,200
2.5	1 "	9.9	7.0	1 "	420,000
2.6	1 "	11.6	8.0	1 "	17,000,000
2.7	1 "	14			

TABLE IV.
Normal Males, November 1914.

Age.	No. weighed.	Weight.	Deviation from normal of July 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
5	15	14.63	+2.18	3.3	24.6
6	24	15.54	-0.04	0.1	19.7
7	24	16.77	-1.31	2.6	17.1
8	24	17.92	-1.44	3.1	13.9
9	24	17.92	-2.71	5.3	15.1
10	24	19.10	-2.09	4.2	12.6
11	24	19.06	-2.75	6.0	13.4
12	24	20.46	-2.19	4.4	13.7
13	24	21.38	-1.93	3.7	12.3
14	24	22.58	-1.38	2.7	10.8
15	24	23.23	-1.05	2.4	9.2
16	24	23.73	-1.02	2.3	8.8
17	24	24.23	-0.98	2.2	7.8
18	23	24.48	-1.13	2.6	7.3
19	24	24.71	-1.10	2.4	8.3
20	23	25.15	-0.95	2.3	7.2
21	23	25.04	-1.24	3.0	7.2
22	23	25.61	-0.45	1.2	6.8
23	23	25.35	-0.99	2.6	6.8
24	23	25.65	-1.17	2.9	7.0
25	23	26.02	-1.03	2.4	6.4
26	22	25.89	-1.05	2.4	7.2
27	23	25.70	-0.85	1.9	7.4
28	22	26.14	-1.05	2.3	7.1
29	23	26.09	-0.99	2.2	7.4
30	18	26.06	-1.17	2.7	7.3
32	23	26.72	-0.89	2.1	7.4
34	23	26.48	-1.23	2.7	7.6
36	23	26.54	-1.41	3.1	7.5
38	23	26.91	-1.29	2.8	7.5
40	23	27.17	-1.10	2.3	7.8
42	23	27.76	-0.52	1.1	7.5
44	23	27.85	-0.77	1.5	7.6
46	23	27.85	-0.70	1.4	7.5
48	22	28.11	-0.11	2.3	7.3
50	22	28.00	-0.88	1.8	7.9
52	22	28.02	-0.66	1.3	7.9
54	22	27.84	-0.83	1.6	7.6
56	20	27.85	-1.47	2.8	8.6
58	16	28.00	-1.10	2.1	7.6
60	16	28.19	-0.89	1.6	7.7

TABLE V.
Normal Males, July 1916.

Age.	No. weighed.	Weight.	Deviation from normal of Nov. 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
5	20	13.25	-1.38	1.9	19.2
6	24	15.04	-0.50	0.9	15.8
7	24	16.83	+0.06	0.1	13.7
8	24	18.08	+0.16	0.4	12.1
9	24	19.00	+1.08	2.3	11.9
10	24	19.63	+0.53	1.2	10.6
11	24	19.96	+0.90	1.9	11.9
12	24	20.44	-0.02	0.0	11.5
13	24	20.92	-0.46	0.9	12.1
14	24	21.31	-1.27	2.7	11.3
15	24	21.83	-1.40	3.0	11.6
16	24	22.15	-1.58	3.5	11.5
17	24	22.42	-1.81	4.1	11.7
18	24	22.58	-1.90	4.5	10.9
19	24	22.71	-2.00	4.6	10.7
20	24	23.02	-2.13	5.2	10.5
21	24	23.10	-1.94	4.8	9.1
22	24	23.42	-2.19	5.3	9.8
23	24	23.49	-1.86	4.6	9.8
24	24	23.63	-2.02	5.3	8.8
25	24	23.85	-2.17	6.0	8.3
26	24	24.10	-1.79	4.4	9.5
27	24	24.31	-1.39	3.6	8.5
28	22	24.31	-1.83	4.5	8.8
29	24	24.65	-1.44	3.6	8.5
30	17	24.91	-1.15	2.5	8.6
32	24	24.88	-1.84	4.5	8.9
34	24	25.21	-1.27	3.0	8.7
36	24	25.69	-0.85	2.1	8.3
38	24	25.52	-1.39	3.3	8.8
40	24	25.83	-1.34	3.1	8.8
42	24	26.00	-1.76	4.2	8.3
44	24	26.06	-1.79	4.3	8.3
46	24	26.56	-1.29	3.1	8.2
48	24	26.35	-1.76	4.1	8.5
50	23	26.85	-1.15	2.6	8.2
52	23	27.20	-0.82	1.8	8.3
54	23	27.61	-0.23	0.5	8.7
56	23	27.54	-0.31	0.6	8.0
58	23	27.57	-0.43	0.9	8.7
60	23	27.39	-0.80	1.7	7.5

TABLE VI.
Normal Females, 1916.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	15	12.43	+2.04	6.2	12.9
5	24	13.67	+1.86	6.0	13.2
6	24	15.08	+0.96	2.6	13.9
7	24	15.98	-0.79	2.1	10.8
8	24	16.94	-1.05	3.0	9.0
9	24	17.50	-1.28	3.6	9.7
10	24	17.73	-1.65	4.6	8.0
11	24	18.19	-1.85	4.9	9.8
12	24	18.42	-1.89	5.1	9.0
13	24	18.56	-2.48	6.4	8.8
14	24	19.23	-1.98	4.9	9.5
15	24	19.42	-2.36	5.6	9.8
16	24	19.90	-2.24	5.6	9.5
17	24	20.19	-2.10	5.1	10.1
18	24	20.06	-2.16	5.4	9.3
19	24	20.31	-2.29	5.6	9.6
20	24	20.88	-1.72	4.4	9.7
21	23	20.85	-2.21	5.0	10.2
22	23	20.89	-2.43	5.5	9.8
23	23	21.22	-2.29	5.1	10.7
24	23	21.39	-2.29	5.3	10.6
25	23	21.59	-2.20	4.6	12.0
26	23	21.76	-2.28	4.5	13.1
27	23	22.00	-2.00	3.9	13.1
28	21	22.71	-0.87	1.8	11.6
29	22	22.77	-1.07	2.0	13.1
30	21	22.83	-1.09	2.2	11.4
32	23	23.11	-1.07	2.1	12.3
34	23	23.17	-1.01	2.0	11.4
36	23	23.65	-1.00	2.0	11.4
38	23	23.80	-1.00	1.8	12.9
40	23	23.93	-1.10	2.0	13.1
42	23	24.48	-0.59	1.0	14.2
44	23	24.00	-1.52	2.4	13.3
46	23	24.24	-1.44	2.5	11.2
48	23	24.07	-1.38	2.4	11.2
50	23	24.04	-1.46	2.6	11.1

TABLE VII.
Normal Females, 1917.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	24	10.27	-2.16	5.3	21.4
5	24	12.15	-1.52	3.7	20.0
6	24	13.73	-1.35	3.3	15.2
7	24	14.96	-1.02	2.7	14.2
8	24	15.29	-1.65	4.7	13.3
9	23	16.28	-1.22	3.4	12.1
10	22	16.64	-1.07	3.1	11.5
11	22	17.39	-0.80	2.2	11.0
12	22	17.75	-0.67	1.8	11.3
13	22	18.36	-0.20	0.6	10.3
14	22	18.61	-0.62	1.6	11.0
15	22	19.05	-0.37	0.9	11.0
16	22	18.80	-1.10	2.4	13.9
17	22	19.55	-0.64	1.5	11.0
18	22	19.66	-0.40	1.0	11.0
19	22	20.05	-0.26	0.6	11.0
20	22	20.59	-0.29	0.7	10.2
21	22	21.05	+0.20	0.5	9.5
22	22	21.48	+0.59	1.4	9.8
23	22	21.70	+0.48	1.1	9.0
24	22	21.68	+0.29	0.7	9.1
25	22	22.25	+0.66	1.3	10.1
26	22	22.77	+1.01	1.9	10.2
27	21	22.81	+0.81	1.6	10.0
28	22	22.70	-0.01	0.0	9.6
29	21	23.24	+0.47	0.8	11.3
30	21	23.71	+0.88	1.7	11.0
32	22	24.16	+1.05	1.8	11.4
34	22	24.23	+1.06	2.0	10.8
36	22	24.50	+0.85	1.5	12.3
38	5	24.50	+0.70	—	—
40*	18	23.19	-0.74	1.2	11.4
42	17	23.59	-0.89	1.5	9.2
44	17	24.47	+0.47	0.9	7.1
46	19	24.89	+0.65	1.2	10.2
48	19	25.13	+1.06	1.9	10.4
50	19	25.53	+1.49	2.9	9.1

* Moved from California to Toronto.

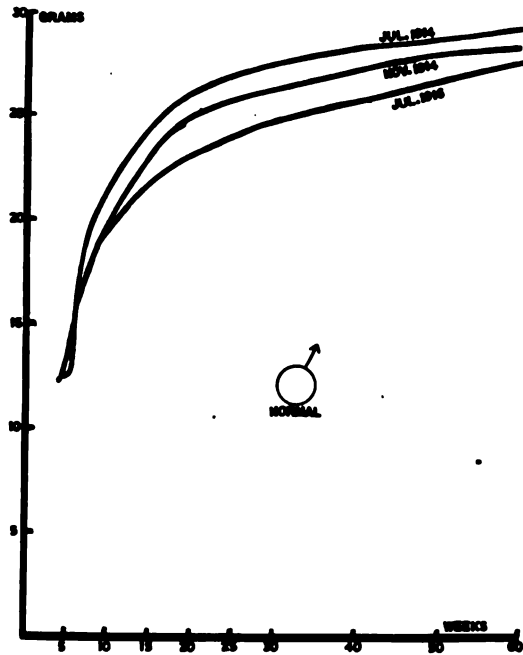


FIG. 3. Progressive changes in the growth curve of normal male white mice from July 1914 to July 1916.

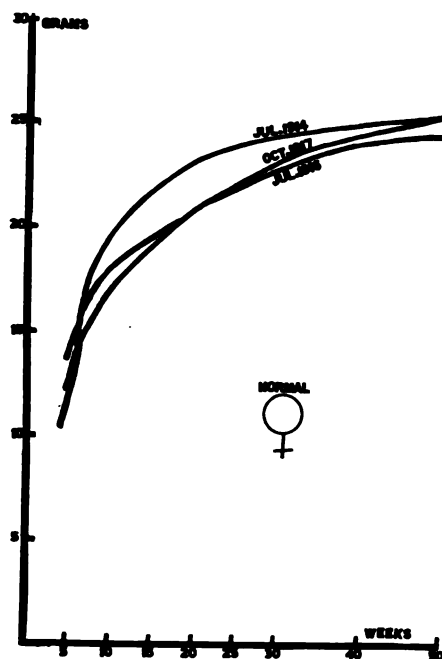


FIG. 4. Progressive changes in the growth curve of normal female white mice from July 1914 to October 1917.

EXPERIMENTAL STUDIES ON GROWTH.

XI. THE GROWTH AND SENESCENCE OF WHITE MICE FED UPON PITUITARY (ANTERIOR LOBE) TISSUE, TETHELIN, EGG LECITHIN, OR CHOLESTEROL.

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General Characteristics of Effects Observed.

In preceding articles of this series¹ data were presented concerning the growth of animals receiving (superadded to a varied and abundant normal diet) specified amounts of pituitary (anterior lobe) tissue, tethelin,² egg lecithin, or cholesterol. The results reported at that time extended only to the 60th week of life and the 56th week of the administration of the substances added to the diet. The conclusions reached were: (1) the pituitary (anterior lobe) tissue in dosage of one-twelfth of an anterior lobe of the ox per mouse per day causes a retardation of growth during the first part of the third or adolescent growth cycle, followed by a secondary or compensatory acceleration; (2) tethelin in dosage of 4 mg. per mouse per day leads to similar results of a much more pronounced character and extent; (3) egg lecithin in dosage of 40 mg. per mouse per day causes a uniform retardation of growth probably originating in a certain degree of toxicity of the very large dose of lecithin or of the choline split off from it during digestion; (4) cholesterol in dosage of 40 mg. per mouse per day causes a pronounced initial retardation and sec-

¹ Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385, 397; xxv, 635, 647.

² Concerning the preparation, properties, and actions of tethelin, a phospholipin derived from the anterior lobe of the pituitary body, see: Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 409; *Endocrinology*, 1917, i, 24. Schmidt, C. L. A., *J. Lab. and Clin. Med.*, 1916-17, ii, 711. Schmidt, C. L. A., and May, E. S., *ibid.*, 1916-17, ii, 708.

ondary acceleration similar in type to that produced by much smaller doses of tethelin.

In the previous reports, however, only the absolute magnitudes of the observed deviations from normality were considered, and no estimate of their real significance in terms of the probable error of the measurements was furnished. In the present experiments the results previously published have been amplified by continuing the observations from the 60th week to natural death, and the observed deviations from the normal have been computed in the only manner which enables us to determine the precise de-

TABLE I.
Summary of Tables II to IX.

Class of animals	Sex.	Average ratio of deviation from normal to probable error of estimate.			
		Weeks.			
		4-30	32-60	62-90	92-120
Pituitary.....	♂	2.1	0.8	1.1	1.8
"	♀	4.0	1.1	0.6	1.2
Tethelin.....	♂	4.2	3.9	3.2	1.0
"	♀	3.4	2.0	2.7	2.2
Lecithin.....	♂	5.0	2.5	4.7	4.5
"	♀	5.2	3.3	3.3	2.7
Cholesterol...	♂	5.7	3.9	4.5	4.0
"	♀	6.5	2.5	3.2	3.3

gree of significance they possess; namely, in terms of the probable error of the estimation of the deviations themselves.³

These extended results confirm in general the conclusions set forth in the earlier reports. They are summarized in Table I, and are shown in detail in Tables II to IX and Figs. 1 to 8.

The greater the value of the ratio of the observed deviations from the normal to the probable error of their estimation, the greater is the significance which attaches to them. From Table III of the preceding article, it will be seen that a deviation which is twice the probable error of the estimation has one chance in

³ Paper X of this series.

5.6 of being accidental, while a deviation which is six times the probable error of the estimation has only one chance in over 19,000 of being accidental in origin. A ratio of less than one indicates that the observed deviation from the normal is more probably accidental than not.

Table I shows that in all classes of animals, whether receiving pituitary tissue, tethelin, lecithin, or cholesterol, the deviations from the normal expressed in probable error units are greatest in the first 30 weeks and thereafter diminish progressively. Evidently all the different classes of animals of the same sex tended to approach a common curve of final equilibrium, the attainment of which was only prevented by the onset of senescence and the loss of weight and mortality which ensue. The substances administered to the animals, therefore, influenced the growth process in the way that catalyzers influence chemical reactions; that is, by affecting the velocity with which equilibrium is attained without affecting the equilibrium itself.

In all instances, except one, the effect of the substance added to the dietary was, during the first 30 weeks, much greater in the females than in the males. The sole exception is that afforded by the tethelin-fed animals in which the effect upon the males was greater than upon the females. This exception affords striking testimony to the reality of the effects observed, for tethelin was only administered to the females for three periods of 1 month each (see Fig. 4), while it was administered to the males daily, without interruption.

Effects of Pituitary (Anterior Lobe) Tissue.

The computation of the deviations in terms of probable error units shows that the effect of the dosage of pituitary tissue administered upon the growth of the male animals was of uncertain significance, since the observed deviations were only from one to two times the probable error. That the deviations from the normal nevertheless were real and due to the administration of the pituitary tissue is evidenced by the much greater effect of the same character upon the female, consisting of retardation during the earlier stages of growth. In both males and females the deviations from the normal after the 30th week are of indeterminate

significance; that is, the growth curves of the normal and of the pituitary-fed animals are, so far as our estimates reveal, identical after 7 months of age. Hence the preliminary retardation of growth has clearly, prior to the 30th week, been succeeded by acceleration.

Effects of Tethelin.

The dosage of pituitary tissue which was administered to the pituitary-fed animals corresponded to a dosage of only about 0.8 mg. of tethelin *per diem*. The effect of 4 mg. of tethelin per day is much more marked and consists of initial retardation followed by marked secondary or compensatory acceleration. The effects are definite in magnitude and remain so until the last period of 92 to 120 weeks (see Table I) when the diminishing number of survivors in both the normal and experimental groups so enhances the probable error of the estimations that marked deviations are requisite for significance.

It remains to inquire whether the acceleration of growth succeeding the 15th week in the tethelin-fed groups is due to the tethelin directly or only indirectly; whether it is due to direct stimulation of the growth process by the tethelin, or to some compensatory process started in the animal itself by the administration, and as a protection against the retarding action of the tethelin.

In our previous reports we subscribed to the former alternative, but we now incline to the latter since we find that by discontinuing the administration of tethelin before the attainment of sexual maturity of the animals the acceleration not only still occurs, but is enhanced. In fact, we have succeeded by this method in producing some very large animals; namely, a number of females weighing over 35 and even over 40 gm. each. The details of these results will be reported later but an idea of their character may be obtained from the curves in Paper XIV of this series.

Effects of Egg Lecithin.

In our earlier reports we considered egg lecithin to be without direct influence upon the growth processes, for the reason, primarily, that the curves of growth of the lecithin-fed animals,

although below normal, remained parallel to the normal curves throughout the first 60 weeks without exhibiting the decisive deformations of contour characteristic of the growth curves of tetelin-fed or cholesterol-fed animals. Our present method of evaluating the results shows that our previous conclusion was incorrect and at the same time reveals the value of the statistical method when applied to investigations of this character.

The deviations from normality in the first 30 weeks are of such magnitude that there is but one chance in 1,300 that they were accidental, nor could any initial error in sampling the material from which the experimental animals were chosen have produced these deviations, for during the first few weeks of lecithin dietary the deviations from the normal increased from three to six times the probable error in the males, and from 0.5 to six times the probable error in the females.

The deviation from normality, although diminishing somewhat (in probable error units) with advancing age, remained significant throughout life.

We incline to the view that the effects of egg lecithin in dosage of 40 mg. daily are of a twofold origin. That there is probably some toxicity is indicated by the indifferent appearance of the animals (described in our previous reports), by the subnormal duration of life (cf. Paper XII), and by their remarkable susceptibility to infectious and degenerative lesions (cf. Paper XIII). This is confirmed by the marked tendency (nearly always present, but especially prominent in the lecithin-fed animals) to abmodality in the frequency-curve of mortality; *i.e.*, the marked divergence between the mean and the median durations of life (cf. Paper XII). The scattering of deaths is not uniform on either side of the greatest frequency, but, on the contrary, the 50 per cent of animals which survive the median duration of life survive to a greater age than we should anticipate from the distribution of the earlier deaths. Hence a small proportion of lecithin-fed animals, notwithstanding the subnormal duration of life of the average, survived to what would constitute a very advanced age even in normal animals.

The egg lecithin employed was a crude preparation, obtained by simply precipitating the ether extract of egg yolks with acetone, draining, and drying without reprecipitation. It must

therefore have consisted of a mixture of lipins. We are inclined to refer the toxicity of the material to the lecithin itself, administered in excessively large dosage, while in view of the crude character of the preparation used and the ease with which lecithin itself is known to be saponified and the consequent unlikelihood of its absorption without alteration, we are inclined to attribute its effects upon the time relations of growth to a substance or substances associated with our preparations, rather than to the lecithin itself.

Effects of Cholesterol.

The effects of cholesterol in dosage of 40 mg. daily are the most marked, and consist, as previously stated, in decided initial retardation followed by acceleration of growth. The deviations from normal are significant throughout life and could not have arisen accidentally.

Possible Errors of Sampling.

The foundation upon which statistical comparisons are based is the assumption, the correctness of which must be verified in each particular instance, that the material subjected to measurement is uniformly variable about a fixed mean, so that successive samples differ from one another only fortuitously and not in consequence of any systematic cause of variation or shift in value of the mean. Now as we have seen in the preceding article the normal animals from which the experimental animals were chosen were varying³ and the mean weight at every age was changing with the lapse of time. It was impossible to start all these feeding experiments simultaneously, involving as they did the use of over 500 animals, and therefore a systematic source of error underlies the results and it becomes necessary to estimate the degree to which it may have affected their verity.

The experiments were begun in the following order: normals (born July 1914), pituitary (born July 1914), lecithin (born August 1914), tethelin, males (born August 1914), cholesterol (born October 1914), tethelin, females (born October 1914). In November to December 1914 a fresh batch of normal males was chosen, which, as explained in the preceding article, were found to

differ from the July normals by deviations averaging only about twice the probable error of the estimate. Moreover, their growth curve showed no trace of deformation of contour such as the concavity between 10 and 20 weeks which characterizes the growth curves of tethelin and cholesterol-fed animals. Quantitatively and qualitatively, therefore, the observed effects could only have been affected by errors of sampling to an insignificant degree.

The reality of the effects observed is, however, furthermore established, in a striking way, by the fact that in all the experiments the effect of the administration (except for the reasons noted above, in the tethelin group) was much greater in the females than in the males. The progressive alteration of the normal with time was very much *less*³ in the female than in the male. The result is therefore the opposite of what it would have been had the observed divergences from normality been due to errors in sampling the material used for measurement.

Effects upon Variability.

The effects of the various substances administered upon the variability of the animals were diverse. In all classes of animals and in both sexes the variability during the early months of growth was depressed in proportion to the diminution of the velocity of growth. In later months, with the exception of the cholesterol-fed groups, the variability of the males remained subnormal, while that of the females regained the normal level or even exceeded it. Exceptions, as stated, were afforded by both sexes of the cholesterol-fed group, which, despite their subnormal weight, became abnormally variable in weight after the first 6 or 7 months. This high variability was probably attributable in part to the disturbances of function arising from extensive deposits of cholesterol in various organs,⁴ and the degenerative lesions which resulted

³ Anitschkow, N., *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 379; 1914, lvii, 201. Chalatow, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85. Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 68; 1915-16, xiii, 60.

The presence of extensive deposits of doubly refracting lipoids in the liver and adrenals of these animals was confirmed by Dr. C. H. Bailey, to whom our thanks are due for examining many specimens of the material.

therefrom in later months (Paper XIII). It has been pointed out in connection with the growth of children⁵ that subnormal velocity of growth, when accompanied by deleterious conditions, leads to enhanced variability, while under favorable conditions it leads to subnormal variability.

In all the different classes of animals, normal as well as experimental, a tendency was displayed for a small group of similar animals to survive to the last weeks. In other words, a certain small number of animals which resembled each other in surviving longest also resembled each other in weight, as though a certain rather definite weight standard, differing in each class of animals, were the most favorable for survival.

Effects upon Senescence.

In all the growth curves (Figs. 1 to 8) senescent loss of weight precedes by a definite interval the average duration of life, and the terminal oscillations of the curve reveal successively steeper descending limbs, showing that senescent loss of tissue is proceeding with augmenting velocity as age increases. The position of the maximum of the growth curve is, however, considerably affected by the administrations, and almost disappears in the curve of the tethelin males (which received tethelin continuously) and in the curves of the lecithin-fed animals. The hump on the normal curve is replaced in these cases by a long, flattened plateau, gradually rising (tethelin males) or falling (lecithin males and females) till the relatively rapid fall due to senescence supervenes. It is probably significant that the greatest flattening of the curve (*i.e.*, slowing of the rate of change of growth velocity) occurred in precisely those groups of animals which displayed the greatest duration of life (tethelin males), or else in which certain exceptional individuals displayed a remarkably enhanced duration of life (lecithin males).

⁵ Robertson, T. B., *Am. J. Physiol.*, 1916, xli, 547.

TABLE II.
Pituitary-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	20	12.78	+0.40	0.8	22.7
5	36	13.13	+0.68	1.7	24.1
6	36	15.86	+0.28	0.6	22.1
7	36	17.38	-0.70	1.5	17.7
8	36	18.60	-0.76	1.6	17.3
9	36	19.54	-1.09	2.3	14.8
10	36	20.03	-1.16	2.3	15.4
11	36	20.58	-1.23	2.7	14.9
12	36	21.79	-0.86	2.0	11.4
13	36	22.36	-0.95	2.1	10.7
14	36	23.00	-0.96	2.1	10.0
15	36	23.06	-1.22	3.0	9.8
16	36	23.67	-1.08	2.5	9.3
17	36	24.11	-1.10	2.5	9.9
18	36	24.17	-1.44	3.3	9.7
19	36	24.57	-1.24	2.8	10.2
20	36	24.63	-1.47	3.5	9.8
21	36	24.99	-1.29	3.0	9.6
22	36	25.35	-0.71	1.7	11.5
23	36	25.85	-0.49	1.3	8.7
24	36	26.19	-0.63	1.4	10.5
25	36	25.92	-1.13	2.5	9.8
26	36	26.11	-0.83	1.8	9.2
27	36	26.11	-0.44	1.0	8.8
28	34	26.29	-0.90	2.0	8.7
29	35	26.23	-0.85	1.9	8.8
30	33	26.48	-0.75	1.9	8.4
32	36	26.68	-0.93	2.2	9.0
34	34	27.35	-0.36	0.8	9.7
36	32	27.33	-0.62	1.4	9.3
38	32	27.55	-0.65	1.4	9.3
40	32	28.03	-0.24	0.5	9.2
42	31	28.05	-0.23	0.4	10.6
44	30	28.32	-0.30	0.6	10.0
46	31	28.24	-0.31	0.6	10.1
48	30	28.05	-0.17	0.3	11.1
50	30	28.47	-0.41	0.8	11.2
52	30	28.58	-0.10	0.2	11.2
54	30	28.92	+0.25	0.4	11.4
56	30	28.90	-0.42	0.7	11.1
58	30	29.35	+0.25	0.5	11.0
60	30	29.42	+0.34	0.6	11.0

TABLE II—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>cks.</i>		<i>gm.</i>			<i>per cent</i>
62	30	29.12	-0.29	0.5	10.6
64	30	29.30	-0.23	0.4	11.3
66	30	29.80	+0.10	0.2	11.4
68	30	29.63	-0.17	0.3	11.4
70	28	29.29	-0.41	0.7	11.9
72	28	29.43	-0.27	0.5	11.0
74	28	29.64	-0.12	0.2	11.5
76	28	29.21	-0.77	1.4	11.2
78	27	29.41	-0.75	1.1	10.2
80	27	29.02	-0.93	1.7	9.8
82	27	29.04	-1.27	2.2	10.1
84	27	29.33	-0.55	0.9	10.2
86	26	28.44	-1.66	2.7	8.2
88	25	28.82	-1.37	2.1	9.0
90	25	28.90	-1.23	2.1	8.6
92	24	28.04	-2.42	4.3	6.5
94	24	28.44	-1.69	3.1	7.9
96	24	28.29	-1.38	2.7	7.8
98	23	28.17	-1.19	2.2	8.4
100	23	28.24	-1.30	2.5	8.1
102	21	28.31	-1.17	1.9	9.2
104	21	27.90	-1.71	3.0	7.5
106	21	28.00	-1.03	1.7	8.1
108	17	27.94	-0.62	1.1	6.7
110	16	27.91	-0.34	0.5	7.6
112	13	28.46	+0.16	0.3	6.3
114	12	27.29	-0.87	1.2	9.1
116	11	27.32	-0.87	1.1	6.8
118	11	27.45	-0.84	1.1	6.7
120	11	27.59	+0.34	0.5	6.3
122	11	27.23	+0.15	0.2	6.2
124	11	27.00	-0.32	0.5	6.6
126	10	26.95	+0.22	0.3	7.9
128	7	26.93	-0.62	0.8	7.9
130	6	25.75	-2.36	2.9	6.5
132	6	26.00	-2.00	1.8	8.1
134	6	25.67	-2.33	—	8.1
136	5	24.90	-2.10	—	7.2
138	4	25.38	-0.12	—	—
140	4	25.13	-0.87	—	—
142	2	26.25	—	—	—
144	2	26.00	—	—	—
146	2	25.50	—	—	—
148	1	26.50	—	—	—

TABLE III.
Pituitary-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	31	9.53	-0.86	3.1	20.5
5	35	11.76	-0.05	0.2	19.6
6	35	13.89	-0.23	0.7	16.1
7	36	14.71	-2.06	5.4	15.8
8	36	16.24	-1.75	4.5	15.1
9	36	17.22	-1.56	4.1	13.1
10	36	17.93	-1.45	3.7	12.1
11	36	18.24	-1.80	5.1	10.5
12	36	18.88	-1.43	4.1	9.3
13	36	19.21	-1.83	4.8	10.3
14	36	19.57	-1.64	4.3	9.4
15	36	19.72	-2.06	5.1	10.4
16	36	19.90	-2.24	6.1	9.3
17	36	20.49	-1.80	4.9	9.6
18	36	20.72	-1.50	3.9	9.7
19	35	21.16	-1.44	3.8	9.7
20	36	21.21	-1.39	3.9	9.1
21	35	21.34	-1.72	4.4	9.6
22	36	21.67	-1.65	4.0	10.2
23	35	21.67	-1.84	4.6	9.9
24	36	22.07	-1.61	4.1	10.2
25	35	22.04	-1.75	4.4	10.4
26	36	22.10	-1.94	4.8	10.5
27	35	22.29	-1.71	4.2	10.3
28	36	22.54	-1.04	2.8	10.0
29	35	22.39	-1.45	3.5	10.3
30	34	22.91	-1.01	2.4	9.6
32	36	23.07	-1.11	2.6	10.8
34	36	23.42	-0.76	1.8	10.8
36	36	23.63	-1.02	2.4	10.9
38	36	24.01	-0.79	1.6	12.2
40	36	24.38	-0.65	1.4	11.8
42	36	24.44	-0.63	1.3	12.4
44	34	24.62	-0.90	1.5	13.1
46	34	24.68	-1.00	1.7	13.2
48	34	24.74	-0.71	1.2	13.7
50	34	25.38	-0.12	0.2	13.3
52	34	25.71	-0.05	0.1	15.4
54	33	25.82	+0.04	0.1	15.2

TABLE III—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
56	33	26.06	+0.06	0.1	15.0
58	33	26.26	±0.00	0.0	15.9
60	31	26.65	+0.53	0.7	14.5
62	31	26.69	+0.29	0.4	16.0
64	31	26.47	-0.17	0.2	16.3
66	30	27.02	+0.37	0.4	16.8
68	29	27.29	+0.74	0.9	16.9
70	29	26.90	+0.77	0.9	17.0
72	29	26.95	+0.39	0.5	16.6
74	29	27.07	+0.31	0.4	16.8
76	27	26.87	-0.37	0.4	15.7
78	27	26.72	-0.67	0.8	13.9
80	27	26.85	-0.79	0.9	16.2
82	27	27.15	-0.24	0.3	17.6
84	26	27.08	-0.31	0.3	17.6
86	25	27.34	-0.55	0.6	17.6
88	24	27.29	-0.48	0.5	14.4
90	23	26.93	-0.89	1.0	15.6
92	22	26.55	-1.43	1.6	11.6
94	22	26.52	-1.01	1.2	11.8
96	21	26.76	-1.27	1.4	13.2
98	20	26.58	-1.31	1.2	13.0
100	20	26.03	-1.39	1.3	13.7
102	20	25.88	-1.74	1.6	12.4
104	20	26.18	-0.88	0.9	12.3
106	17	24.97	-1.60	1.7	9.0
108	17	24.88	-1.94	1.8	10.5
110	17	24.82	-1.48	1.3	11.3
112	15	25.27	-1.43	1.4	8.9
114	15	25.30	+0.87	1.1	10.7
116	10	25.50	+0.93	1.0	12.3
118	8	24.50	+0.21	0.2	14.3
120	6	25.08	+0.94	0.8	13.9
122	6	24.75	-0.18	0.1	16.0
124	5	22.90	-2.03	1.8	14.4
126	5	22.50	-2.00	1.7	13.8
128	5	22.50	-3.30	2.7	15.8

TABLE IV.

Tethelin-Fed Males. Continuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	7	13.36	+0.98	—	—
5	20	13.05	+0.60	1.3	21.8
6	24	14.69	-0.89	1.9	18.2
7	24	16.54	-1.54	3.4	14.6
8	24	18.21	-1.15	2.6	12.0
9	24	18.98	-1.65	3.8	10.2
10	24	19.50	-1.69	3.6	10.8
11	24	19.92	-1.89	4.4	10.9
12	24	20.56	-2.09	4.8	10.6
13	24	20.88	-2.43	5.1	10.5
14	24	20.88	-3.08	6.2	11.2
15	24	21.21	-3.07	7.3	9.1
16	24	21.90	-2.85	6.5	8.4
17	24	22.65	-2.56	6.0	8.0
18	24	23.21	-2.40	5.7	7.3
19	24	23.79	-2.02	4.7	7.2
20	24	24.35	-1.75	4.6	6.3
21	24	24.52	-1.76	4.4	6.2
22	23	24.87	-1.19	3.1	7.6
23	23	24.61	-1.73	4.5	6.4
24	23	24.91	-1.91	4.7	7.7
25	23	25.33	-1.72	4.1	6.7
26	23	25.30	-1.64	3.6	8.1
27	23	25.59	-0.96	2.1	8.0
28	23	25.46	-1.73	3.8	7.4
29	23	25.63	-1.45	3.2	7.9
30	22	25.70	-1.53	3.7	7.2
32	23	26.00	-1.61	3.9	9.6
34	23	26.22	-1.49	3.2	8.8
36	23	26.02	-1.93	4.5	6.9
38	23	26.24	-1.96	4.4	7.4
40	23	26.43	-1.84	4.5	5.4
42	23	26.54	-1.74	3.9	5.8
44	23	26.50	-2.12	4.3	6.9
46	23	26.74	-1.81	3.7	7.0
48	23	26.78	-1.44	3.7	6.9
50	23	26.52	-2.36	4.8	7.8
52	23	26.98	-1.70	3.6	7.2
54	23	27.63	-1.04	2.1	7.0
56	23	27.72	-1.60	3.1	7.7
58	23	27.33	-1.77	3.8	6.7
60	23	26.93	-2.15	4.3	7.3

TABLE IV—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
62	23	27.46	-1.95	3.9	7.0
64	23	27.61	-1.92	3.8	7.2
66	23	27.22	-2.48	4.8	7.9
68	23	28.07	-1.73	3.4	7.9
70	23	28.11	-1.59	3.1	7.8
72	23	28.07	-1.63	3.1	7.4
74	23	27.96	-1.80	3.4	8.4
76	23	28.52	-1.46	3.0	7.7
78	23	28.17	-1.99	3.7	8.2
80	22	28.61	-1.34	2.3	9.2
82	21	28.50	-1.81	3.3	7.7
84	21	28.12	-1.76	3.0	7.9
86	21	28.38	-1.72	2.7	8.8
88	21	28.21	-1.98	3.1	8.4
90	19	29.32	-0.81	1.3	7.9
92	19	28.82	-1.64	2.6	8.4
94	19	28.89	-1.24	2.1	8.2
96	18	28.86	-0.81	1.4	8.7
98	18	28.83	-0.53	0.8	10.1
100	18	28.78	-0.76	1.4	8.2
102	18	28.58	-0.90	1.5	8.2
104	18	29.00	-0.61	1.0	8.5
106	18	28.78	-0.25	0.4	9.2
108	18	28.83	+0.27	0.5	7.9
110	18	28.72	+0.47	0.7	7.9
112	18	28.75	+0.45	0.7	7.8
114	18	28.72	+0.56	0.9	7.3
116	15	28.07	-0.12	0.2	6.7
118	13	28.08	-0.21	0.3	7.7
120	13	27.62	+0.37	0.5	7.3
122	13	27.73	+0.65	0.9	8.4
124	12	27.96	+0.64	0.9	7.6
126	12	27.75	+1.02	1.7	7.1
128	10	27.05	-0.50	0.8	3.8
130	9	26.89	-1.22	1.7	2.6
132	9	26.72	-1.28	1.3	3.9
134	7	26.57	-1.43	—	5.1
136	6	26.58	-0.92	—	5.7
138	5	26.50	+1.00	—	—
140	4	27.13	+1.13	—	—
142	3	26.67	—	—	—
144	2	26.00	—	—	—

TABLE V.
Tethelin-Fed Females. Discontinuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	12	10.96	+0.57	0.9	27.6
5	16	13.44	+1.63	3.4	19.6
6	16	14.94	+0.82	1.5	19.4
7	16	15.78	-0.99	1.9	16.1
8	16	16.19	-1.80	4.1	12.6
9	16	16.63	-2.15	4.8	12.4
10	16	17.03	-2.35	4.8	13.2
11	16	17.53	-2.51	4.6	15.7
12	16	17.66	-2.65	4.6	16.6
13	16	18.97	-2.07	3.8	14.1
14	16	19.72	-1.49	2.9	11.9
15	16	20.53	-1.25	2.4	11.4
16	16	20.19	-1.95	4.0	11.2
17	16	20.59	-1.70	3.4	11.5
18	16	20.31	-1.91	4.0	10.7
19	16	20.59	-2.01	3.9	11.9
20	16	20.59	-2.61	4.0	12.1
21	16	21.09	-1.97	3.9	11.2
22	16	21.13	-2.19	4.2	11.1
23	16	21.47	-2.04	3.8	12.2
24	16	21.78	-1.90	3.4	12.7
25	16	21.75	-2.04	3.6	12.8
26	16	22.00	-2.04	3.4	13.5
27	16	21.84	-2.16	3.7	13.6
28	16	22.22	-1.36	2.3	14.2
29	14	22.36	-1.48	2.3	14.1
30	16	22.38	-1.54	2.3	15.4
32	16	22.59	-1.59	2.4	15.4
34	16	23.06	-1.12	1.6	15.7
36	16	22.88	-1.77	2.6	15.7
38	16	23.44	-1.36	1.9	15.6
40	16	23.53	-1.50	2.0	16.9
42	16	23.53	-1.54	1.9	17.9
44	16	23.95	-1.58	1.8	18.8
46	16	23.59	-2.09	2.5	17.8
48	16	23.50	-1.95	2.4	17.1
50	16	24.09	-1.41	1.7	17.6
52	16	24.41	-1.35	1.6	16.6
54	16	24.41	-1.37	1.6	17.2
56	16	24.56	-1.44	1.6	17.1
58	16	24.38	-1.88	2.1	16.7
60	16	24.31	-1.81	2.0	16.8
62	16	24.28	-2.12	2.4	16.8

TABLE V—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
64	16	24.34	-2.30	2.5	16.7
66	16	24.16	-2.29	2.5	16.1
68	16	24.06	-2.49	2.9	14.7
70	16	24.06	-2.07	2.3	15.4
72	15	25.07	-1.49	1.6	16.3
74	15	24.70	-2.06	2.2	15.5
76	15	24.90	-2.34	2.5	15.7
78	15	24.73	-2.66	2.8	15.5
80	15	24.77	-2.87	2.9	16.4
82	15	23.93	-3.46	3.6	16.0
84	14	24.21	-3.54	3.6	16.6
86	13	24.73	-3.16	3.1	16.1
88	13	24.85	-2.92	2.6	17.9
90	13	24.54	-3.28	3.2	16.8
92	11	24.91	-3.07	2.8	15.1
94	11	25.41	-2.12	2.1	12.5
96	10	24.85	-3.18	3.2	12.6
98	8	24.56	-3.33	2.7	14.3
100	8	24.25	-3.17	2.5	14.2
102	8	24.56	-3.06	2.4	15.0
104	8	24.19	-2.87	2.5	13.1
106	8	24.00	-2.57	2.3	12.0
108	8	24.06	-2.76	2.2	13.4
110	8	23.44	-2.86	2.4	10.8
112	8	23.13	-3.57	3.3	10.3
114	8	23.31	-1.12	1.2	13.0
116	7	22.79	-1.78	1.9	12.0
118	7	23.36	-0.93	0.9	12.2
120	6	23.00	-1.14	1.2	16.1
122	6	23.08	-1.85	1.8	12.8
124	6	23.17	-1.76	1.8	12.5
126	5	23.80	-0.70	0.6	13.7
128	5	22.60	-3.20	3.0	12.8
130	5	22.70	-3.10	—	13.4
132	5	22.90	-2.20	—	13.3
134	5	22.50	-3.00	—	11.1
136	4	22.13	-1.87	—	—
138	4	21.75	-2.75	—	—
140	4	21.88	-2.75	—	—
142	4	21.50	-3.38	—	—
144	3	23.33	-0.67	—	—
146	2	22.50	-2.00	—	—
148	2	22.50	-1.50	—	—

TABLE VI.
Lecithin-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	18	10.86	-1.52	3.2	23.1
5	36	11.53	-0.92	2.6	23.2
6	36	13.11	-2.47	5.9	21.7
7	36	15.07	-3.01	6.7	19.4
8	36	16.56	-2.80	5.9	18.5
9	36	17.69	-2.94	6.2	15.8
10	36	19.13	-2.06	4.4	13.7
11	36	20.06	-1.75	4.4	11.4
12	36	20.50	-2.15	5.5	10.2
13	36	21.10	-2.21	5.0	9.8
14	36	21.49	-2.47	5.6	9.4
15	36	22.17	-2.11	5.2	9.9
16	36	22.22	-2.53	6.2	8.9
17	36	22.60	-2.61	6.4	8.5
18	36	23.25	-2.36	5.6	8.7
19	36	23.72	-2.09	5.1	7.5
20	36	24.04	-2.06	5.4	8.0
21	36	24.17	-2.11	5.3	8.2
22	36	24.86	-1.20	3.5	7.6
23	36	24.57	-1.77	4.8	7.9
24	36	24.43	-2.39	6.1	8.7
25	34	24.50	-2.55	6.1	8.6
26	36	25.10	-1.84	4.4	8.4
27	34	24.97	-1.58	3.8	7.4
28	35	25.26	-1.93	4.4	8.8
29	33	25.27	-1.81	4.1	8.8
30	34	25.74	-1.49	3.7	8.8
32	36	26.04	-1.57	3.8	9.6
34	36	26.32	-1.39	3.2	8.5
36	35	26.86	-1.09	2.5	8.8
38	35	26.84	-1.36	3.0	8.8
40	33	27.02	-1.25	2.6	8.3
42	33	27.23	-1.05	2.1	9.5
44	33	27.15	-1.47	2.8	10.0
46	33	27.17	-1.38	2.7	10.1
48	32	27.47	-0.75	1.6	9.1
50	31	27.44	-1.44	2.9	9.7
52	31	27.76	-0.92	1.9	9.3
54	30	28.07	-0.60	1.2	8.7
56	30	27.82	-1.50	2.8	9.0

TABLE VI—Continued.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
58	30	27.93	-1.17	2.5	7.8
60	28	27.75	-1.33	2.6	8.1
62	28	27.50	-1.91	3.9	7.5
64	28	27.93	-1.60	3.3	7.6
66	28	27.86	-1.84	3.8	7.4
68	27	27.44	-2.36	4.8	7.3
70	26	27.81	-1.89	3.9	7.8
72	25	27.66	-2.04	4.1	6.4
74	25	27.60	-2.16	4.1	8.1
76	24	27.50	-2.48	5.2	7.6
78	22	27.20	-2.96	6.4	5.1
80	22	27.56	-2.39	4.9	6.5
82	22	27.16	-3.15	5.8	7.5
84	22	26.98	-2.90	5.4	6.0
86	20	27.00	-3.10	5.2	6.7
88	19	27.21	-2.98	5.0	6.0
90	18	27.39	-2.74	4.8	6.4
92	18	27.36	-3.10	5.6	5.2
94	17	26.91	-3.22	5.9	6.6
96	16	26.63	-3.04	5.7	6.8
98	16	26.93	-2.43	6.1	6.5
100	16	26.31	-3.23	6.2	7.6
102	16	26.81	-2.67	4.8	6.3
104	16	26.38	-3.23	5.9	6.1
106	16	26.28	-2.75	4.7	6.3
108	15	26.99	-1.57	2.9	6.3
110	13	25.65	-2.60	4.2	6.3
112	13	26.42	-1.88	2.9	6.6
114	13	26.00	-2.16	3.5	6.1
116	11	26.27	-1.92	2.4	8.1
118	11	25.32	-2.97	4.2	5.6
120	10	25.50	-1.75	2.3	6.0
122	9	24.89	-2.19	3.4	5.9
124	9	24.83	-2.49	3.9	4.8
126	9	24.67	-2.06	3.8	5.0
128	9	24.39	-3.16	4.6	5.6
130	9	24.28	-3.83	5.0	6.0
132	9	24.39	-3.61	3.5	7.0
134	9	23.56	-4.44	—	5.9
136	9	23.50	-4.00	—	7.1
138	6	24.17	-1.33	—	4.1

TABLE VI—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
140	6	24.08	-1.92	—	4.6
142	5	25.00	—	—	4.6
144	4	24.50	—	—	1.5
146	4	24.00	—	—	2.5
148	3	24.17	—	—	—
150	3	24.50	—	—	—
152	3	24.67	—	—	—
154	3	24.67	—	—	—
156	3	20.83	—	—	—
158	3	21.67	—	—	—
160	2	20.25	—	—	—
162	2	21.50	—	—	—
164	1	21.50	—	—	—
166	1	21.00	—	—	—

TABLE VII.
Lecithin-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
5	31	11.42	-0.39	1.4	15.8
6	36	13.35	-0.77	2.7	12.7
7	36	15.04	-1.73	5.4	9.7
8	36	16.47	-1.52	4.8	8.8
9	36	17.00	-1.78	5.6	8.6
10	36	17.32	-2.06	5.6	9.6
11	36	18.08	-1.96	5.9	8.2
12	36	18.67	-1.64	4.8	8.6
13	35	18.71	-2.33	6.7	8.2
14	35	19.26	-1.95	5.6	7.6
15	35	19.72	-2.06	5.4	8.9
16	35	20.03	-2.11	5.9	8.4
17	35	20.26	-2.03	5.7	8.5
18	35	20.50	-1.72	4.6	9.0
19	35	20.77	-1.83	4.9	8.6
20	35	20.64	-1.96	5.8	8.5
21	35	21.03	-2.03	5.3	8.8
22	35	21.13	-2.19	5.6	9.1
23	35	21.31	-2.20	5.6	9.0
24	35	21.27	-2.41	6.3	9.4
25	35	21.34	-2.45	6.3	9.4
26	35	21.66	-2.38	6.3	8.9
27	35	21.61	-2.39	6.0	9.8
28	33	21.92	-1.66	4.5	9.8
29	35	22.06	-1.78	4.3	10.2
30	29	22.34	-1.58	3.6	10.6
32	34	22.29	-1.89	4.4	11.2
34	33	22.56	-1.62	3.7	10.9
36	33	22.88	-1.77	4.1	10.8
38	33	22.82	-1.98	4.2	11.4
40	33	22.97	-2.06	4.6	11.2
42	33	23.00	-2.07	4.1	12.1
44	33	23.29	-2.23	3.9	12.9
46	32	23.20	-2.48	4.4	13.6
48	32	24.02	-1.43	2.4	14.2
50	31	24.08	-1.42	2.4	14.4
52	31	24.60	-1.16	1.8	14.0
54	29	24.55	-1.23	1.8	14.7
56	29	24.28	-1.72	2.5	14.1
58	29	24.69	-1.57	2.3	13.5
60	28	24.09	-2.03	2.9	13.1
62	28	23.73	-2.67	3.9	12.4

TABLE VII—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
64	28	24.34	-2.30	3.2	12.9
66	28	24.82	-1.63	2.1	13.1
68	28	23.86	-2.69	3.5	14.4
70	27	24.61	-1.52	1.9	14.4
72	25	23.26	-3.30	4.2	15.0
74	22	24.41	-2.35	2.9	14.0
76	22	24.77	-2.47	3.0	14.5
78	22	24.93	-2.46	2.9	14.3
80	22	24.73	-2.91	3.4	14.4
82	22	24.57	-2.82	3.4	14.4
84	21	24.19	-3.56	4.3	14.5
86	21	24.31	-3.58	3.9	15.8
88	21	24.52	-3.25	3.5	15.7
90	20	24.55	-3.27	3.5	17.6
92	19	24.79	-3.19	3.1	17.8
94	19	24.32	-3.21	3.1	18.4
96	19	24.05	-3.98	3.8	18.8
98	18	24.53	-3.36	3.0	17.3
100	16	23.88	-3.54	3.1	16.6
102	16	23.75	-3.87	3.5	14.9
104	13	22.96	-4.10	3.9	13.8
106	13	23.38	-3.19	3.0	14.8
108	12	22.75	-4.07	3.6	12.4
110	11	22.50	-3.80	3.2	13.3
112	9	22.94	-3.76	3.5	10.5
114	8	22.75	-1.68	1.9	10.9
116	8	22.88	-1.69	1.8	12.8
118	8	22.69	-1.60	1.8	11.3
120	8	22.69	-1.45	1.7	11.1
122	7	21.93	-3.00	3.3	10.0
124	6	21.00	-3.93	4.6	10.6
126	5	21.40	-3.10	3.1	12.0
128	5	21.30	-4.50	4.5	12.3
130	5	21.20	-4.60	4.5	12.9
132	5	21.20	-3.90	—	12.6
134	4	20.50	-5.00	—	10.2
136	4	20.25	-3.75	—	11.2
138	3	21.50	-3.00	—	—
140	2	22.00	-2.63	—	—
142	2	21.75	-3.13	—	—
144	2	21.50	-3.00	—	—
146	1	19.00	-5.50	—	—

TABLE VIII.
Cholesterol-Fed Males.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
5	22	12.84	+0.39	1.0	18.1
6	33	14.06	-1.52	3.6	19.3
7	34	15.85	-2.23	5.2	16.2
8	34	16.88	-2.48	5.6	15.5
9	34	16.79	-3.84	7.8	17.9
10	34	17.41	-3.78	7.3	18.4
11	34	17.85	-3.96	8.2	18.2
12	34	18.96	-3.69	7.7	16.1
13	34	20.46	-2.85	5.8	13.5
14	34	21.37	-2.59	5.3	12.4
15	34	21.88	-2.40	5.2	13.3
16	34	22.03	-2.72	5.7	12.9
17	32	22.20	-3.01	6.7	11.0
18	34	23.10	-2.51	5.3	11.5
19	32	22.55	-3.26	7.4	9.9
20	34	23.37	-2.73	6.2	11.0
21	30	23.47	-2.81	6.5	9.1
22	34	24.19	-1.87	4.7	10.5
23	30	24.02	-2.32	5.7	9.4
24	29	24.43	-2.39	5.3	10.3
25	29	24.24	-2.81	6.3	8.9
26	29	24.48	-2.46	5.3	10.5
27	29	24.16	-2.39	5.3	9.2
28	29	25.00	-2.19	4.6	10.4
29	29	24.59	-2.49	5.5	9.1
30	28	24.77	-2.46	5.1	11.2
32	34	25.34	-2.27	4.9	11.1
34	34	25.62	-2.09	4.2	11.3
36	34	25.66	-2.29	4.7	11.8
38	33	26.18	-2.02	4.3	9.9
40	27	26.06	-2.21	4.1	11.8
42	32	26.19	-2.09	3.9	11.5
44	32	26.27	-2.35	4.4	10.4
46	32	27.00	-1.55	2.9	11.7
48	32	26.84	-1.38	2.9	9.4
50	32	26.83	-2.05	4.2	9.3
52	32	26.78	-1.90	3.8	10.5
54	32	27.50	-1.17	2.2	9.9
56	32	26.98	-2.34	4.3	9.8

TABLE VIII—Continued.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
58	32	26.78	-2.32	4.4	11.3
60	32	26.88	-2.20	4.0	10.8
62	32	27.06	-2.35	4.4	10.1
64	32	26.98	-2.55	4.8	10.2
66	32	26.95	-2.75	5.0	10.8
68	32	27.06	-2.74	5.1	10.9
70	31	27.23	-2.47	4.7	10.2
72	31	27.23	-2.47	4.3	10.9
74	31	27.31	-2.45	4.4	11.1
76	29	27.59	-2.39	4.5	10.8
78	29	26.93	-3.23	5.7	11.1
80	29	27.41	-2.54	4.6	10.3
82	28	27.71	-2.60	4.3	10.9
84	27	27.13	-2.75	4.3	11.8
86	27	27.28	-2.82	4.1	12.4
88	27	27.67	-2.52	3.7	11.3
90	27	27.31	-2.82	4.2	12.3
92	27	27.00	-3.46	5.2	12.1
94	26	26.79	-3.34	5.0	13.7
96	26	26.81	-2.86	4.5	13.0
98	25	26.46	-2.90	4.5	13.4
100	23	26.22	-3.32	5.4	14.4
102	22	26.37	-3.11	4.3	13.9
104	22	26.43	-3.18	4.5	13.6
106	21	26.43	-2.60	3.7	12.4
108	19	26.24	-2.32	3.5	12.0
110	19	26.05	-2.20	2.9	12.8
112	18	25.64	-2.66	3.3	14.0
114	18	25.78	-2.38	3.0	13.7
116	17	24.94	-3.25	3.7	13.8
118	15	24.40	-3.89	4.2	15.5
120	13	24.92	-2.33	2.5	15.4
122	10	25.40	-1.68	1.5	17.0
124	9	25.56	-1.76	1.8	17.3
126	8	23.94	-2.79	3.0	14.1
128	7	24.36	-3.19	3.0	14.0
130	5	25.50	-2.61	2.6	9.6
132	4	25.63	-2.37	1.6	12.4
134	4	24.38	-3.62	—	10.3
136	3	24.50	-3.00	—	—

TABLE VIII—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
138	2	24.25	-1.25	—	—
140	2	24.25	-1.75	—	—
142	2	24.00	—	—	—
144	2	24.25	—	—	—
146	2	24.25	—	—	—
148	2	22.50	—	—	—
150	2	22.75	—	—	—
152	2	22.25	—	—	—
154	1	24.00	—	—	—
156	1	23.00	—	—	—
158	1	22.00	—	—	—
160	1	22.00	—	—	—

TABLE IX.
Cholesterol-Fed Females.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
5	14	13.14	+1.33	2.5	21.5
6	35	13.10	-1.02	3.2	15.6
7	35	13.90	-2.87	7.5	16.5
8	35	14.90	-3.09	8.1	15.4
9	35	15.10	-3.68	8.8	17.8
10	35	15.54	-3.84	9.4	15.2
11	35	16.06	-3.98	9.3	18.1
12	35	17.10	-3.21	9.5	16.1
13	35	17.73	-3.31	9.2	16.9
14	35	18.46	-2.75	6.2	14.7
15	35	18.77	-3.01	6.8	13.7
16	35	19.21	-2.93	7.0	13.3
17	33	19.30	-2.99	7.3	12.8
18	34	19.66	-2.56	6.2	12.8
19	33	19.65	-2.95	7.0	12.3
20	34	20.12	-2.48	6.4	12.1
21	33	20.24	-2.82	6.4	12.9
22	34	20.35	-2.97	6.7	12.4
23	33	20.48	-3.03	6.8	12.4
24	33	20.80	-2.88	6.3	14.3
25	33	20.94	-2.85	6.1	14.5
26	33	21.24	-2.80	5.8	14.7
27	33	21.32	-2.68	5.5	14.8
28	33	21.56	-2.02	4.2	15.7
29	33	21.62	-2.22	4.4	15.2
30	34	22.07	-1.85	3.4	16.6
32	33	22.08	-2.10	3.8	17.3
34	32	22.28	-1.90	3.2	18.4
36	32	22.41	-2.24	3.7	19.9
38	32	22.89	-1.91	2.9	20.4
40	32	22.88	-2.14	3.2	21.0
42	31	22.90	-2.17	3.0	22.8
44	30	23.30	-2.22	2.8	23.1
46	29	24.14	-1.54	1.9	23.0
48	29	23.86	-1.59	1.9	23.4
50	28	23.86	-1.64	2.0	23.0
52	27	24.06	-1.70	2.0	22.4
54	27	24.61	-1.17	1.3	21.9
56	27	24.30	-1.70	1.9	23.1
58	26	24.13	-2.13	2.3	23.0
60	26	24.35	-1.77	2.0	21.1
62	25	24.66	-1.74	1.9	22.3

TABLE IX—*Concluded.*

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
64	25	24.38	-2.26	2.4	21.7
66	25	24.28	-2.17	2.2	21.6
68	23	23.91	-2.64	3.3	15.5
70	23	23.87	-2.26	2.8	15.5
72	23	24.24	-2.32	2.7	16.9
74	23	24.04	-2.72	3.2	15.8
76	23	23.91	-3.33	3.9	16.8
78	23	23.96	-3.43	4.0	16.0
80	22	24.05	-3.59	3.9	17.9
82	20	24.15	-3.24	3.4	18.5
84	19	24.16	-3.59	3.7	19.1
86	18	24.06	-3.83	3.7	19.3
88	16	24.31	-3.46	3.4	16.5
90	16	24.38	-3.44	3.6	16.2
92	13	24.31	-3.67	3.3	17.4
94	13	24.50	-3.03	3.0	14.8
96	11	24.05	-3.98	3.5	17.1
98	10	22.55	-5.34	4.6	14.7
100	10	23.30	-4.12	3.3	15.9
102	10	23.00	-4.62	3.9	14.2
104	10	23.45	-3.61	3.3	13.3
106	10	23.25	-3.32	3.0	13.7
108	10	22.65	-4.17	3.6	12.9
110	9	22.22	-4.08	3.4	13.1
112	9	22.61	-4.09	3.6	13.0
114	9	22.44	-1.99	2.2	13.0
116	7	22.50	-2.07	2.3	12.2
118	7	21.29	-3.00	3.0	13.1
120	6	21.08	-3.06	3.3	11.9
122	6	21.50	-3.43	3.1	14.6
124	6	21.25	-3.68	3.9	12.3
126	6	21.58	-2.92	2.9	13.0
128	5	22.30	-3.50	3.7	10.9
130	5	22.00	-3.80	—	11.2
132	4	21.25	-3.85	—	6.4
134	4	21.13	-4.37	—	7.8
136	4	21.00	-3.00	—	8.4
138	4	20.38	-4.12	—	6.1
140	4	20.38	-4.25	—	5.6
142	4	20.63	-4.25	—	4.7
144	4	19.25	-5.25	—	7.1
146	3	19.50	-5.00	—	—
148	1	19.50	-4.50	—	—
150	1	16.50	-7.50	—	—

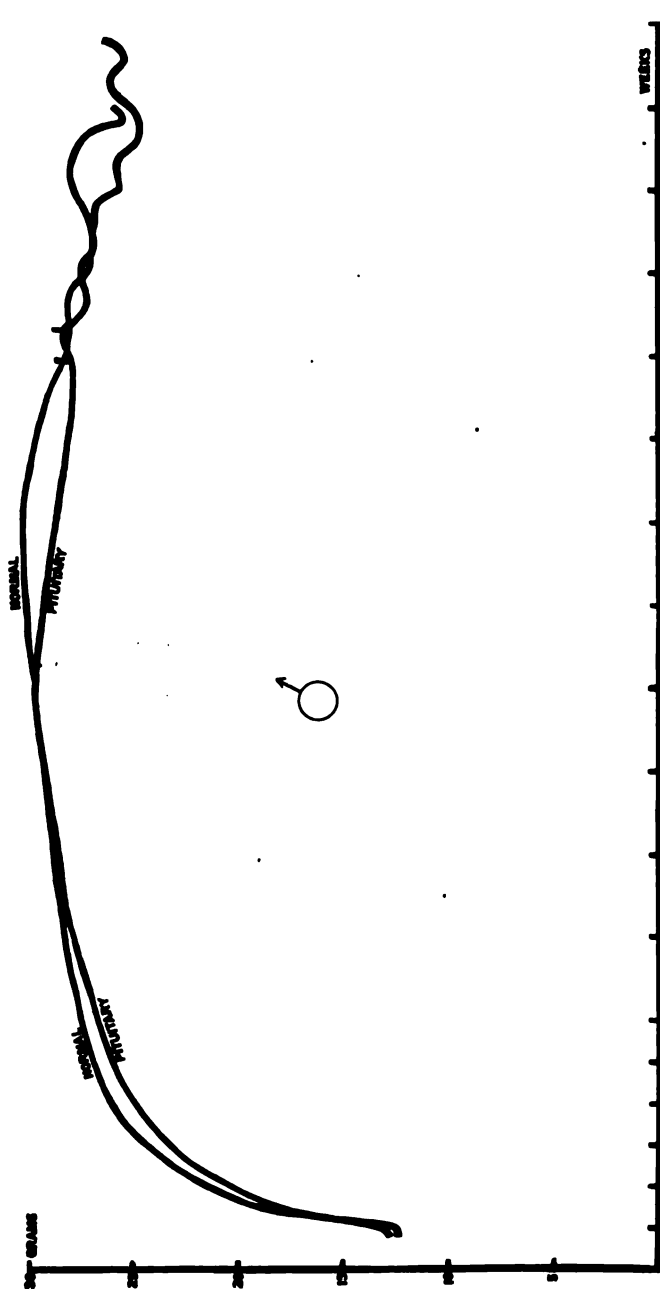


FIG. 1. Influence of pituitary (anterior lobe) tissue upon the growth of male white mice. Dosage one-twelfth of an ox gland per mouse per day. The vertical cross mark indicates average duration of life.

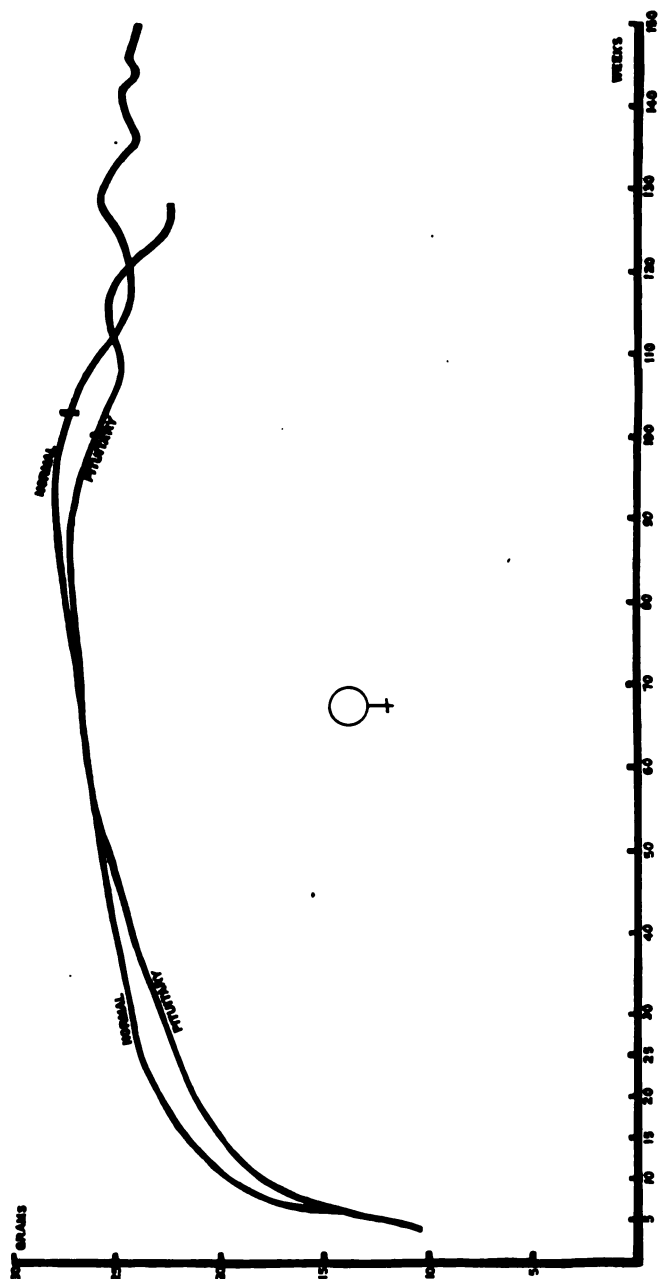


FIG. 2. Influence of pituitary (anterior lobe) tissue upon the growth of female white mice. Dosage one-twelfth of an ox gland per mouse per day. The vertical cross mark indicates average duration of life.

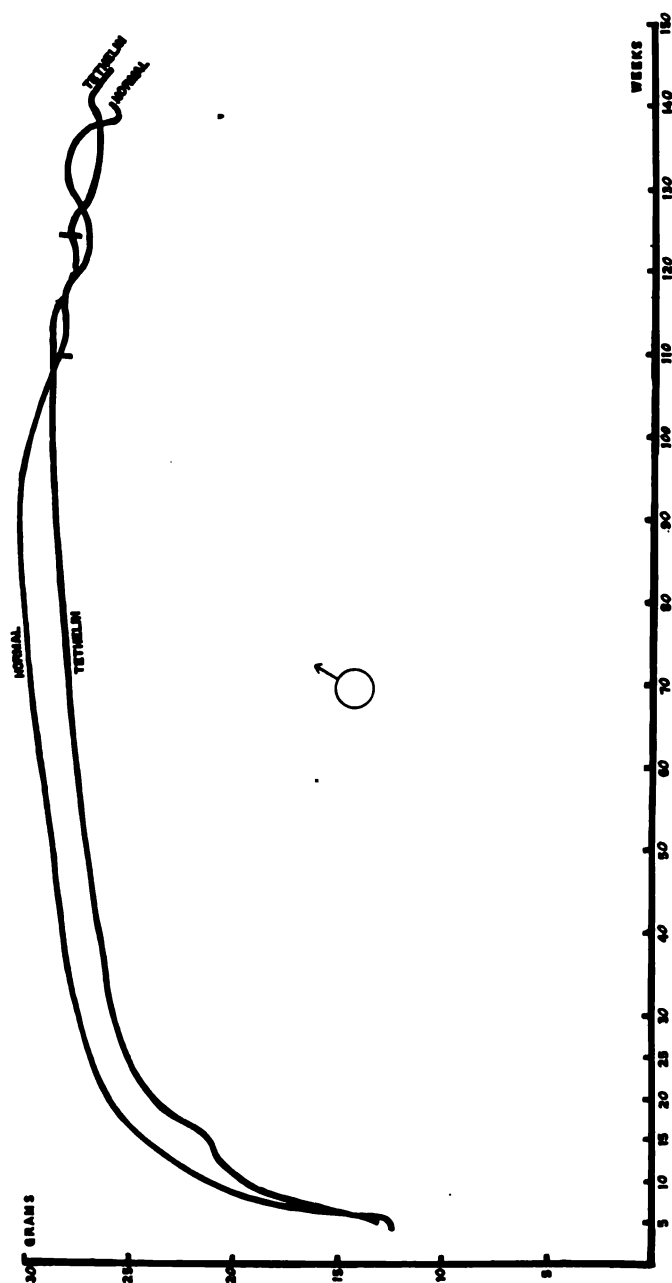


FIG. 3. Influence of tethelin upon the growth of male white mice. Dosage 4 mg. per day. The vertical cross mark indicates average duration of life.

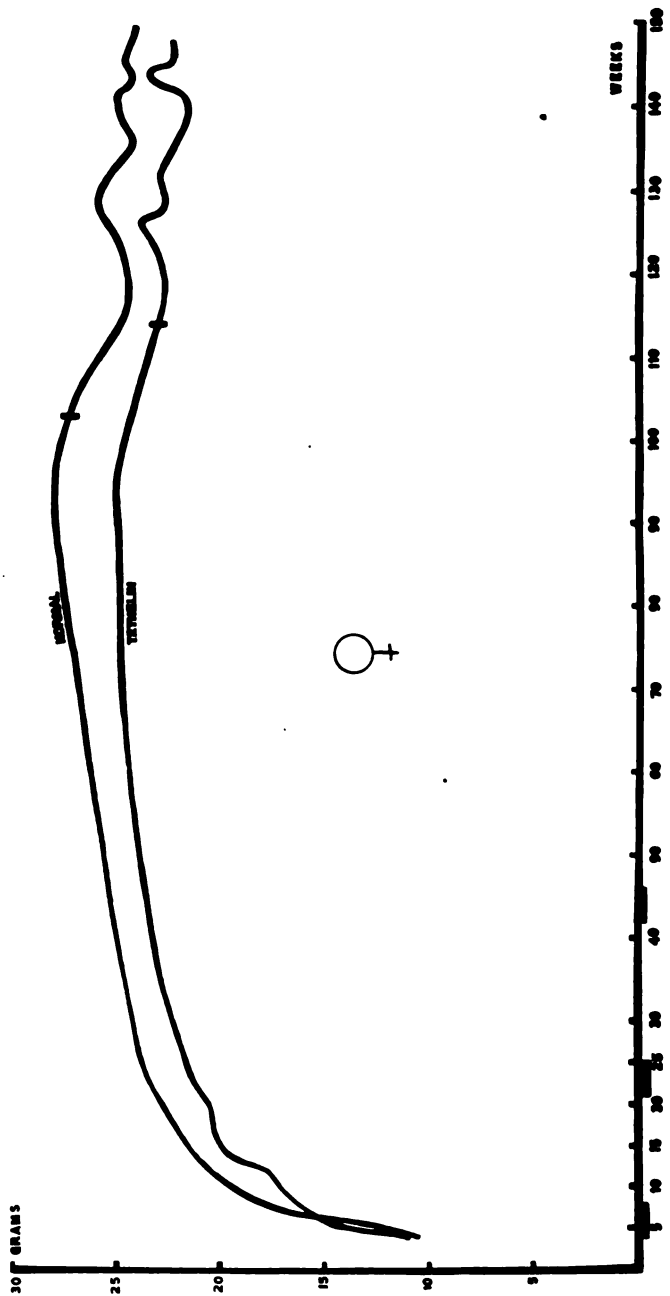


FIG. 4. Influence of tethelin upon the growth of female white mice. Administration of 4 mg. per day in three periods of 4 weeks each, indicated by the heavily shaded portions of the base line. The vertical cross mark indicates average duration of life.

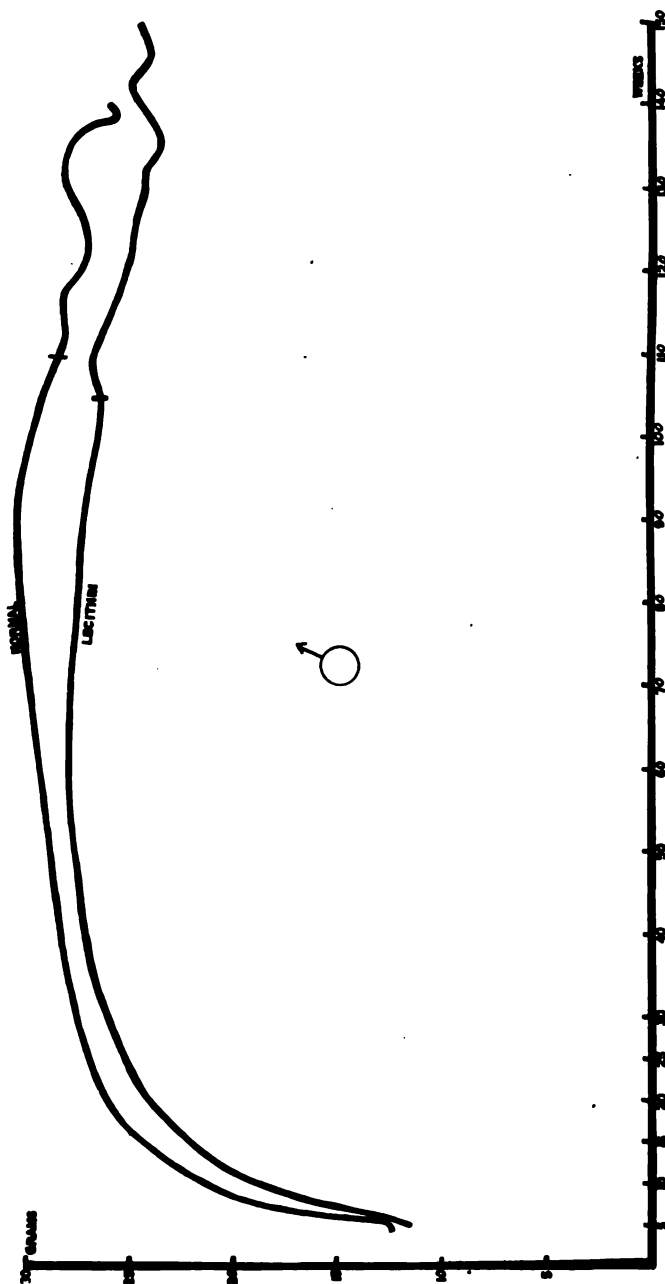


FIG. 5. Influence of lecithin upon the growth of male white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

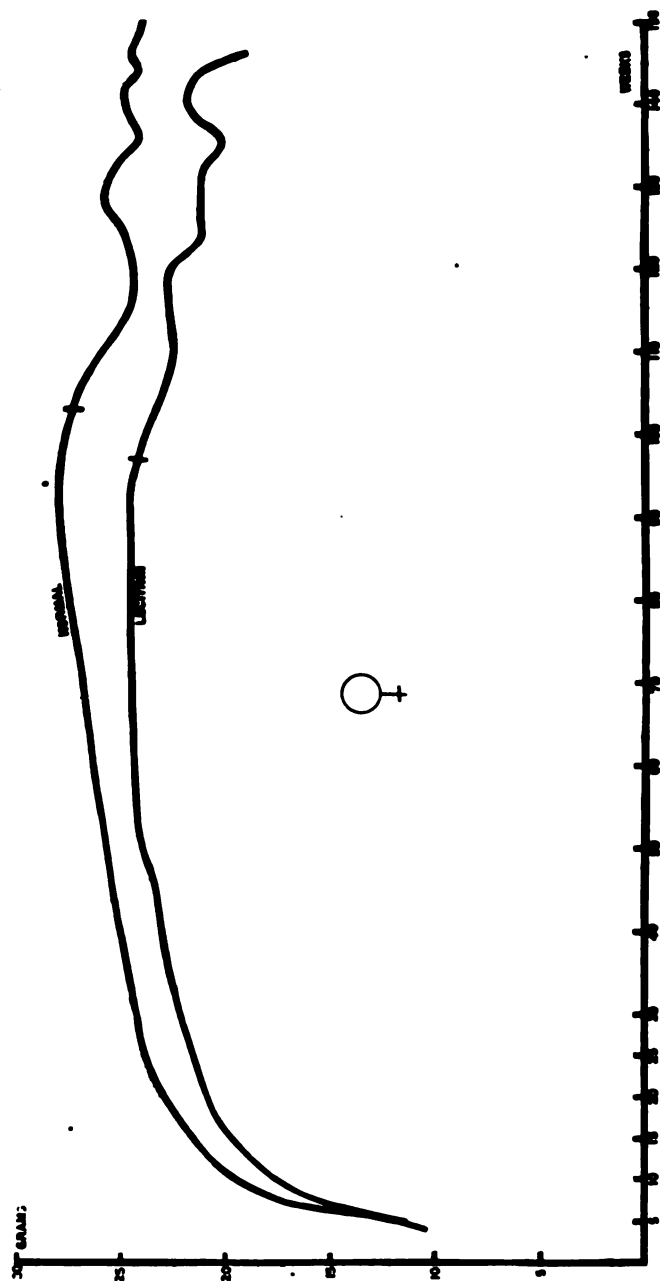


FIG. 6. Influence of lecithin upon the growth of female white mice. Dosage 40 mg. per day. The vertical orom mark indicates average duration of life.

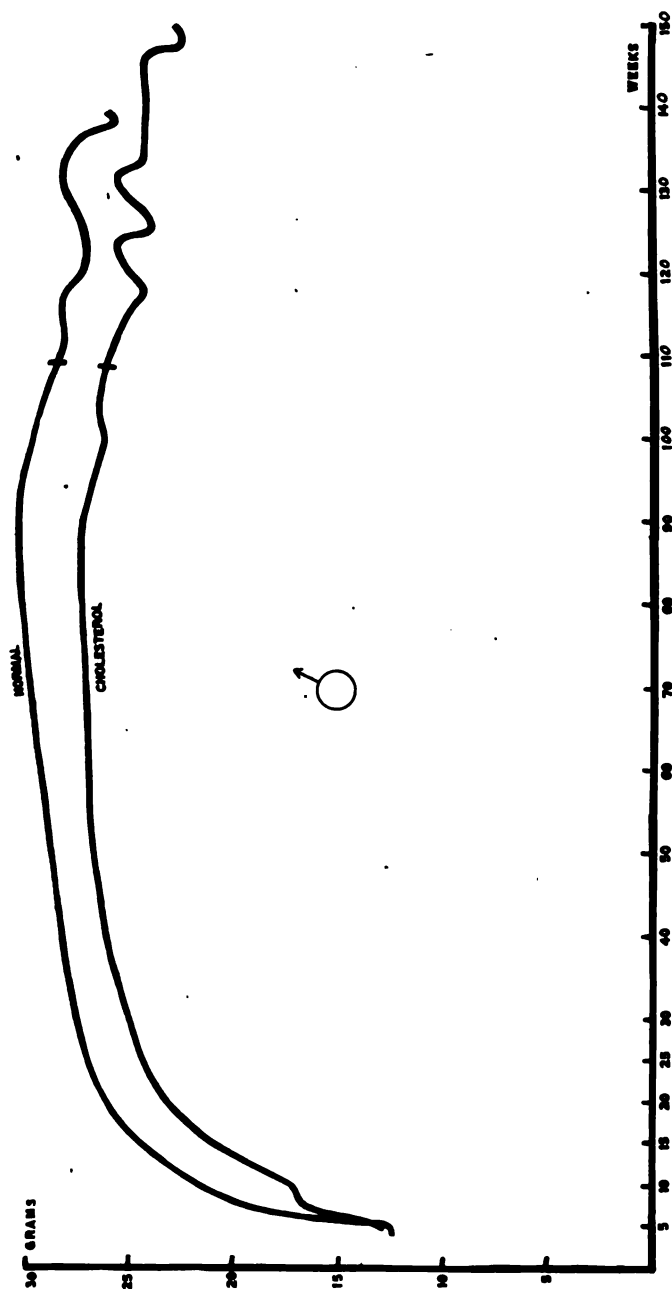


FIG. 7. Influence of cholesterol upon the growth of male white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

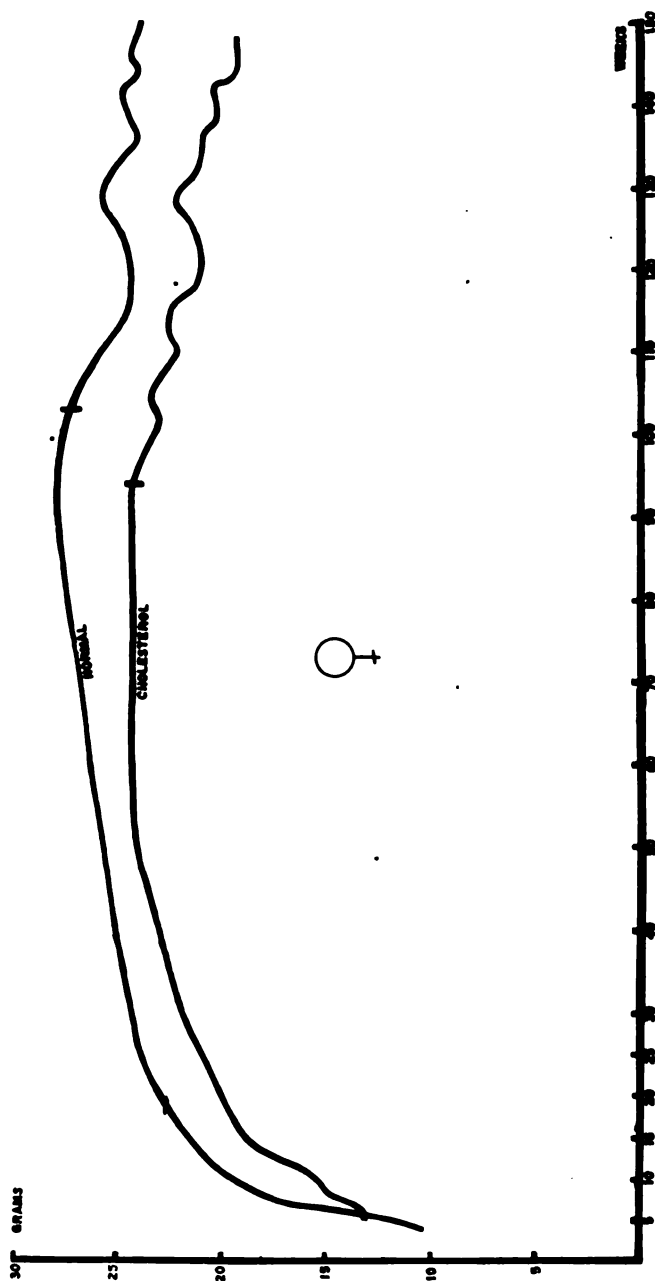


Fig. 8. Influence of cholesterol upon the growth of female white mice. Dosage 40 mg. per day. The vertical cross mark indicates average duration of life.

EXPERIMENTAL STUDIES ON GROWTH.

XII. THE INFLUENCE OF PITUITARY GLAND (ANTERIOR LOBE) TISSUE, TETHELIN, EGG LECITHIN, AND CHOLESTEROL UPON THE DURATION OF LIFE OF THE WHITE MOUSE.

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Methods of Estimation.

Previous estimates of the duration of life of animals, such as those cited by Friedenthal,¹ are for the most part based upon single or relatively few observations, and the duration of life is frequently estimated to be that of the longest lived animal observed. So far as we are aware, no statistical estimation of the life duration of animals has ever been attempted.

According to Mitchell² mice of various species in captivity live for from 16 to 20 months, being presumably grown animals when first introduced to captivity. He quotes Metchnikoff, without detailing the evidence, to the effect that mice may attain the age of 6 years, an estimate which is certainly exaggerated. According to Wood,³ the "expectation of life" for a mouse is 2½ years, but he does not enumerate the data upon which he bases this estimate, which, as we shall see, is somewhat in excess of the correct figure.

All the animals used in the experiments reported in Papers I to VI and Paper XII of this series were retained and the dietary administrations continued,⁴ under exactly the same conditions as

¹ Friedenthal, H., *Zentr. Physiol.*, 1911, xxiv, 321.

² Mitchell, P. C., *Proc. Zool. Soc. London*, 1911, pt. 2, 469.

³ Wood, F. C., *J. Am. Med. Assn.*, 1916, lxvi, 94.

⁴ The administration of cholesterol to the cholesterol-fed animals was, however, discontinued at the end of the 2nd year owing to our inability to procure cholesterol on the market, and lack of time and facilities for producing the large quantities of cholesterol required.

those reported in Paper I,³ throughout their lives. The dosage of pituitary tissue was one-twelfth of an ox gland (anterior lobe) per mouse per day; that of tethelin was 4 mg. per day, and that of lecithin and of cholesterol was 40 mg. per day.

When possible death was allowed to occur naturally; but occasionally, when it appeared that valuable material for postmortem examination might otherwise be lost natural death was anticipated by a few hours or at most a few days, by etherization.

In estimating the part played by senescence in determining the duration of life, deaths which are essentially accidental must be excluded. Such deaths include those due to epidemic infections. The technique adopted in these experiments practically excluded epidemic infections from among the causes of mortality. In only two instances were epidemic infections encountered; namely, when six normal females in one nest developed pneumonia following one case which was not isolated promptly, and when a whole cage of animals (twenty-four) developed a subacute infection of the lungs and nasal epithelium. In the former instance the animals were destroyed and their deaths regarded as accidental and not included in the estimate of the duration of life. In the second instance the whole experiment affected was terminated and its results were rejected.

The majority of accidental deaths occurred in the first few months of life, and in the first few weeks after the transfer of the animals from the breeding cages to the experimental cages, sporadic cases of β -paratyphoid frequently developed. Such animals were killed, and their nests and cages sterilized. Fatal injuries due to fighting also occurred among the young males. For these reasons all deaths prior to 210 days, when the animals may be regarded as adult, are regarded as accidental and rejected in the estimation of the average duration of life.

On the other hand, *sporadic* infections occurring subsequently to 210 days are not regarded as accidental, but are considered to have been determined by the condition and **general resistance** of the animals, so that death due to local or **generalized infections** occurring in single animals was probably occasioned **not by exceptional exposure to infection**, but by **lack of resistance to for-**

³ Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, **xxiv**, 347.

merly acquired and chronic latent infections, resulting from impairment of the vitality of the tissues by senescence. It was frequently observed, particularly in the case of infections of the genitourinary organs in males, that injuries from fighting would be received early in life and would heal completely, to all external appearance. Late in life, usually at an advanced age (*i.e.*, exceeding the mean duration of life), abscesses would form at the spot previously injured. It was inferred that a localized infection had been present from the date of injury, but that the general resistance of the animals had been sufficiently strong to prevent the infection from making headway until senescence and the consequent impairment of resistance gave it an opportunity to proceed and to terminate the life of the animal.

Results.

The results obtained are summarized in Table I and are shown in detail in Tables II to XI and Figs. 1 to 10. In the case of the pituitary tissue and of the lecithin and cholesterol administration the deviations of the mean duration of life from the normal lie within or almost within the probable error of estimation (about 1 month).

In the two groups of animals which received tethelin, however, the duration of life was greatly extended, exceeding the normal by 99 days, or 13 per cent of the normal duration of life in the males which received tethelin continuously, and by 81 days, or 11 per cent of the normal duration of life in the females which received tethelin intermittently in three periods of 1 month each prior to the 30th week.

The experiments on both sexes confirm each other. Since the determinations were carried out in both sexes in the same manner and yet independently, the chance of both observed deviations from normal being accidental in origin may be estimated by multiplying the separate chances of their being accidental. In this way we find that the deviations from the normal duration of life shown by the pituitary-fed and cholesterol-fed animals were more probably accidental than not, and that those displayed by the lecithin-fed animals were almost as probably accidental as not (1 to 1.4). In the case of the tethelin-fed groups, on the

contrary, there is only one chance in 150 that the deviations from the normal duration of life can have been accidental. The correlation of the magnitude of the deviation with the dosage of tethelin administered to the males and to the females respectively adds strong confirmation to this conclusion.

Since none of the ten groups of animals investigated, with the exception of the two tethelin-fed groups, shows any deviation

TABLE I.
Summary of Tables II to XII.

Class of animals.	Average duration of life.	Deviation from normal.	Ratio of deviation to probable error.	Chance that observed deviation was accidental.
Males.				
	<i>days</i>	<i>days</i>		
Normal.....	767	—	—	—
Pituitary.....	792	+25	0.76	1 to 0.64
Cholesterol.....	764	— 3	0.10	1 “ 0.06
Lecithin.....	731	—36	0.94	1 “ 0.90
Tethelin.....	866	+99	3.00	1 “ 22.25
Females.				
Normal.....	719	—	—	—
Pituitary.....	704	—15	0.48	1 to 0.34
Cholesterol.....	658	—61	1.72	1 “ 3.06
Lecithin.....	677	—42	1.27	1 “ 1.55
Tethelin.....	800	+81	2.25	1 “ 6.75
Chances that the results obtained with both sexes were accidental.				
Pituitary.....				1 to 0.2
Cholesterol.....				1 “ 0.2
Lecithin.....				1 “ 1.4
Tethelin.....				1 “ 150.2

from the normal duration of life which may not be regarded as lying within the experimental error of the estimation, we may regard the normal, pituitary-fed, lecithin-fed, and cholesterol-fed animals as forming collectively a series of control animals and estimate from this increased number the normal duration of life with a probable error reduced to approximately one-half. In this way we find that the prolongation of life of the tethelin-fed

males beyond the average of all other classes of males was 103 days, while that of the tethelin-fed females was 108 days, and the chance that both these deviations from the norm thus established were accidental is one in 11,000.

In every group of animals there is a tendency, more or less marked, to bimodality of the frequency curve of death, or curve of the rate of death at varying ages. This tendency is, however, exaggerated in both the groups of tethelin-fed animals, so that two periods of mortality are separated by a period during which the death rate sank to zero. The smaller proportion of the animals died relatively early in life; the larger proportion died in the later period of mortality. The prolongation of the mean duration of life is attributable to the remarkable longevity of the latter group.

The *median* duration of life, or age at which 50 per cent of the animals had expired, was determined graphically from the curve of total deaths by determining the point of intersection with the straight line $y = 50$ per cent. In every case except the cholesterol-fed males and the pituitary-fed females the median duration of life is less than the average duration of life, implying that the animals which survive beyond the median duration of life are to a slight extent selected animals, not differing fortuitously, but to some extent in kind from those which die earlier. This tendency towards asymmetry of the distribution of deaths is slight, the deviation of the median from the mean only definitely exceeding the probable error of the estimation in two instances (cholesterol-fed females and lecithin-fed males). In the case of the tethelin-fed females the median duration of life was indeterminate, because it fell within the period of zero mortality when a single death would have affected the estimate by no less than 100 days.

Although the lecithin-fed animals displayed a diminished rather than an enhanced viability, yet a small group of males attained extraordinary longevity, 9 per cent surviving to over 1,100 days, as compared with 0 per cent of normals, 3 per cent of each of the pituitary- and cholesterol-fed groups, and 4 per cent of the tethelin-fed group. Had we relied solely upon the longevity of the longest lived individual in estimating the duration of life, therefore, we should have been led to select lecithin as the dietary addition most favorable to prolongation of existence,

whereas, the statistical investigation reveals that it is actually unfavorable. The females did not plainly exhibit any similar tendency, and we remain uncertain whether the remarkable longevity of the small group of males referred to was fortuitous in origin, or attributable to the lecithin or to other substances contained in the crude preparation of lecithin employed.

TABLE II.
Mortality of Normal Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
200-300	2	6.0	6.0	241
300-400	0	0.0	6.0	—
400-500	0	0.0	6.0	—
500-600	4	12.5	18.5	555
600-700	4	12.5	31.0	652
700-800	6	19.0	50.0	731
800-900	5	16.0	66.0	847
900-1,000	9	28.0	94.0	952
1,000-1,100	2	6.0	100.0	1,021
Total No. of animals.....				32
Average duration of life.....				767 days
Standard deviation of duration of life.....				200 "
Probable error of average.....				±24 "
Variability of the duration of life.....				26 per cent
Median duration of life (50 per cent dead).....				731 days
Probable error of median.....				±30 "

TABLE III.
Mortality of Normal Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
200-300	1	3	3	293
300-400	2	6	9	348
400-500	2	6	15	495
500-600	5	16	31	545
600-700	3	9	40	684
700-800	9	28	68	760
800-900	5	16	84	845
900-1,000	1	3	87	967
1,000-1,100	4	13	100	1,050

Total No. of animals.....	32
Average duration of life.....	719 days
Standard deviation of duration of life.....	204 "
Probable error of average.....	±24 "
Variability of the duration of life.....	28 per cent
Median duration of life (50 per cent dead).....	710 days
Probable error of median.....	±30 "

TABLE IV.
Mortality of Pituitary-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
300-400	1	3	3	330
400-500	1	3	6	484
500-600	2	6	12	523
600-700	3	10	22	636
700-800	8	26	48	745
800-900	9	29	77	853
900-1,000	3	10	87	917
1,000-1,100	3	10	97	1,033
1,100-1,200	1	3	100	1,181

Total No. of animals.....	31
Average duration of life.....	792 days
Standard deviation of duration of life.....	180 "
Probable error of average.....	±22 "
Variability of the duration of life.....	23 per cent
Median duration of life (50 per cent dead).....	750 days
Probable error of median.....	±27 "

TABLE V.
Mortality of Pituitary-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
300-400	2	6	6	388
400-500	5	14	19	446
500-600	3	9	28	563
600-700	3	9	37	634
700-800	7	20	57	751
800-900	12	34	91	843
900-1,000	3	9	100	926
Total No. of animals.....				35
Average duration of life.....				704 days
Standard deviation of duration of life.....				177 "
Probable error of average.....				±20 "
Variability of the duration of life.....				25 per cent
Median duration of life (50 per cent dead).....				715 days
Probable error of median.....				±25 "

TABLE VI.
Mortality of Tethelin-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
500-600	2	9	9	568
600-700	3	13	22	644
700-800	0	0	22	—
800-900	7	30	52	842
900-1,000	5	22	74	940
1,000-1,100	5	22	96	1,025
1,100-1,200	1	4	100	1,124
Total No. of animals.....				23
Average duration of life.....				866 days
Standard deviation of duration of life.....				157 "
Probable error of average.....				±22 "
Variability of the duration of life.....				18 per cent
Median duration of life (50 per cent dead).....				830 days
Probable error of median.....				±28 "

TABLE VII.
Mortality of Tethelin-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
400-500	1	6	6	496
500-600	1	6	12	596
600-700	6	38	50	659
700-800	0	0	50	—
800-900	3	19	69	859
900-1,000	1	6	75	984
1,000-1,100	4	25	100	1,051

Total No. of animals.....	16
Average duration of life.....	800 days
Standard deviation of duration of life.....	158 " "
Probable error of average.....	±27 "
Variability of the duration of life.....	20 per cent
Median duration of life (50 per cent dead).....	indeterminate

TABLE VIII.
Mortality of Lecithin-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
300-400	2	6.0	6.0	348
400-500	4	12.5	19.0	435
500-600	6	19.0	37.5	552
600-700	4	12.5	50.0	640
700-800	3	9.0	59.0	765
800-900	4	12.5	72.0	848
900-1,000	4	12.5	84.0	973
1,000-1,100	2	6.0	91.0	1,031
1,100-1,200	3	9.0	100.0	1,149

Total No. of animals.....	32
Average duration of life.....	731 days
Standard deviation of duration of life.....	243 "
Probable error of average.....	±29 "
Variability of the duration of life.....	33 per cent
Median duration of life (50 per cent dead).....	640 days
Probable error of median.....	±36 "

TABLE IX.
Mortality of Lecithin-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days.</i>				<i>days</i>
200-300	1	3	3	247
300-400	2	6	9	374
400-500	5	15	24	456
500-600	4	12	35	521
600-700	5	15	50	658
700-800	7	21	70	746
800-900	5	15	85	846
900-1,000	3	9	94	960
1,000-1,100	2	6	100	1,022

Total No. of animals.....	34
Average duration of life.....	677 days
Standard deviation of duration of life.....	202 "
Probable error of average.....	±23 "
Variability of the duration of life.....	30 per cent
Median duration of life (50 per cent dead).....	658 days
Probable error of median.....	±29 "

TABLE X.
Mortality of Cholesterol-Fed Males.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
200-300	1	3	3	293
300-400	1	3	6	329
400-500	1	3	9	490
500-600	4	11	20	556
600-700	5	14	34	678
700-800	6	17	51	768
800-900	11	31	83	861
900-1,000	4	11	94	936
1,000-1,100	1	3	97	1,077
1,100-1,200	1	3	100	1,125

Total No. of animals.....	35
Average duration of life.....	764 days
Standard deviation of duration of life.....	178 "
Probable error of average.....	±20 "
Variability of the duration of life.....	23 per cent
Median duration of life (50 per cent dead).....	765 days
Probable error of median.....	±25 "

TABLE XI.
Mortality of Cholesterol-Fed Females.

Age-group.	No. of deaths.	Per cent of total deaths.	Total per cent of deaths.	Average age at death.
<i>days</i>				<i>days</i>
200-300	2	6	6	263
300-400	2	6	12	357
400-500	4	13	25	449
500-600	4	13	38	555
600-700	9	28	66	652
700-800	2	6	72	754
800-900	4	13	85	842
900-1,000	1	3	87	925
1,000-1,100	4	13	100	1,030

Total No. of animals.....

32

Average duration of life.....

658 days

Standard deviation of duration of life.....

220 "

Probable error of average.....

±26 "

Variability of the duration of life.....

33 per cent

Median duration of life (50 per cent dead).....

595 days

Probable error of median.....

±33 "

TABLE XII.
Proportion of Animals Surviving to Various Ages.

Class of animals.	Percentage surviving to									
	Days.									
	300	400	500	600	700	800	900	1,000	1,100	
Males.										
Normal.....	94	94	94	81.5	69	50	34	6	0	
Pituitary.....	100	97	94	88	78	52	23	13	3	
Cholesterol.....	97	94	91	80	66	49	17	6	3	
Lecithin.....	100	94	81	62.5	50	41	28	16	9	
Tethelin.....	100	100	100	91	78	78	48	26	4	
Females.										
Normal.....	97	91	85	69	60	32	16	13	0	
Pituitary.....	100	94	81	72	63	43	9	0	0	
Cholesterol.....	94	88	75	62	34	28	15	13	0	
Lecithin.....	97	91	76	65	50	30	15	6	0	
Tethelin.....	100	100	94	88	50	50	31	25	0	

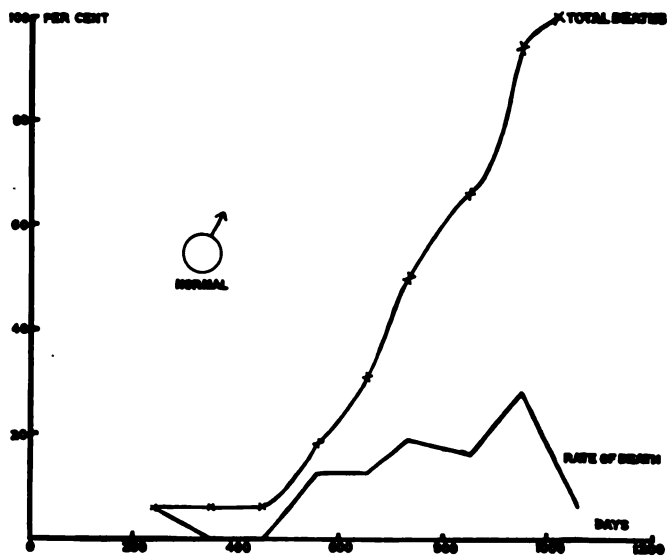


FIG. 1. Mortality curves for normal male white mice.

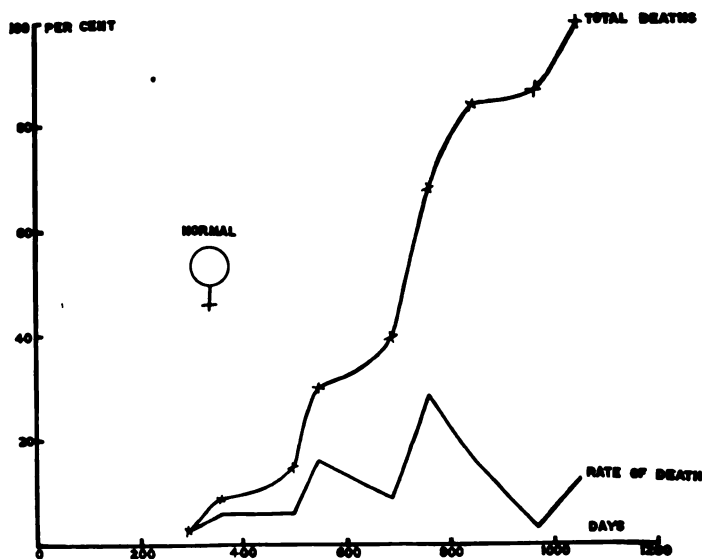


FIG. 2. Mortality curves for normal female white mice.

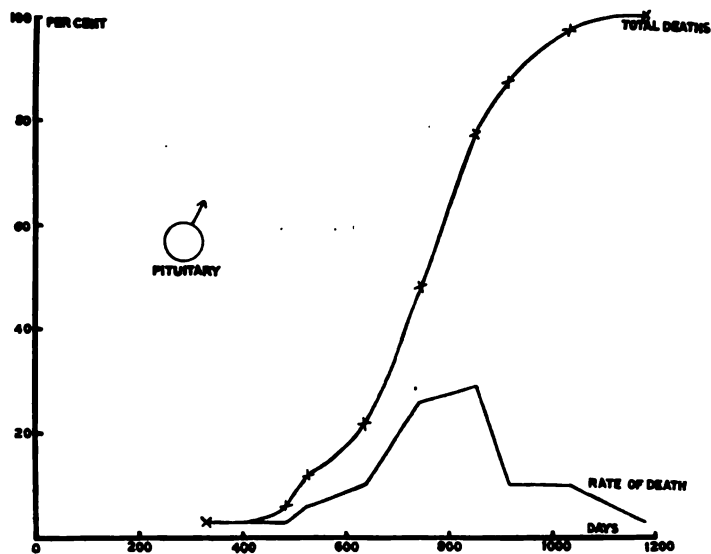


FIG. 3. Mortality curves for pituitary-fed male white mice.

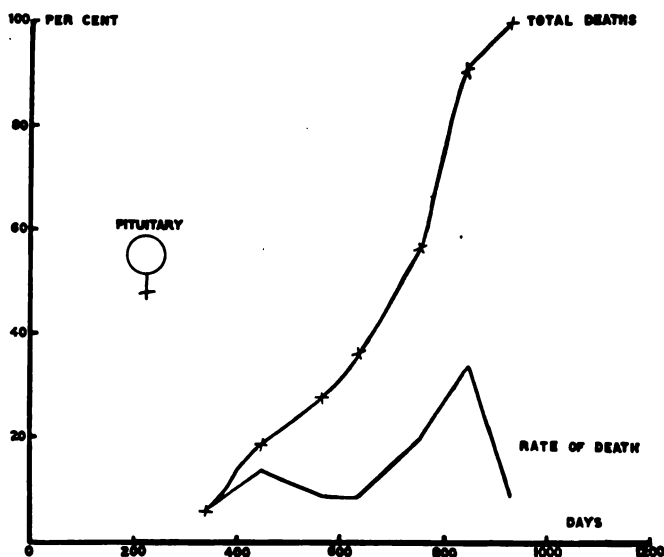


FIG. 4. Mortality curves for pituitary-fed female white mice.

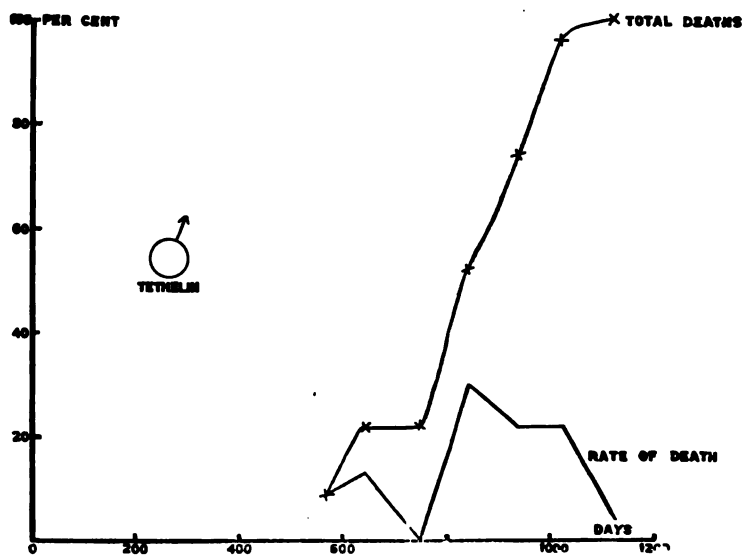


FIG. 5. Mortality curves for tethelin-fed male white mice.

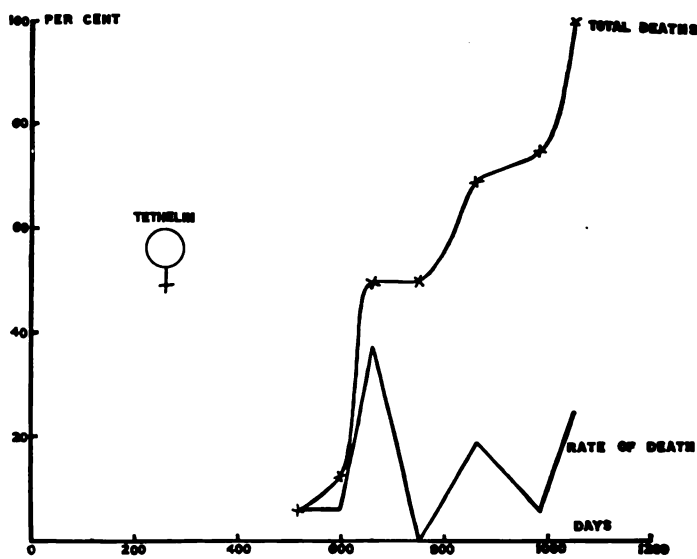


FIG. 6. Mortality curves for tethelin-fed female white mice.

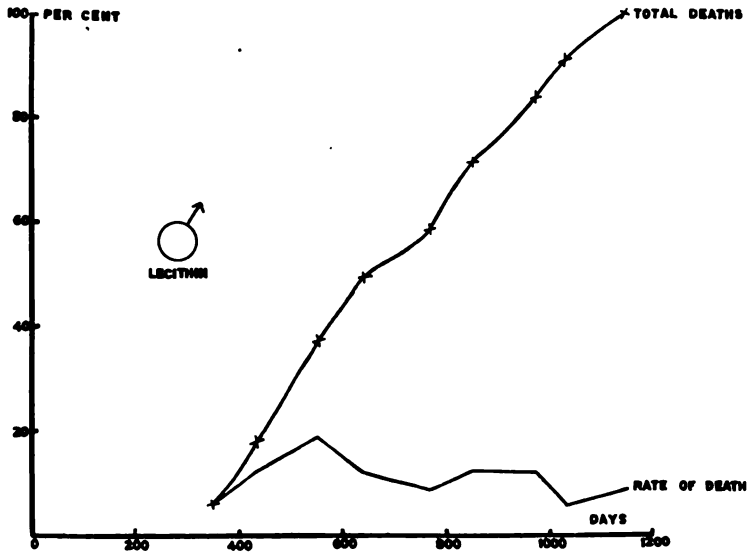


FIG. 7. Mortality curves for lecithin-fed male white mice.

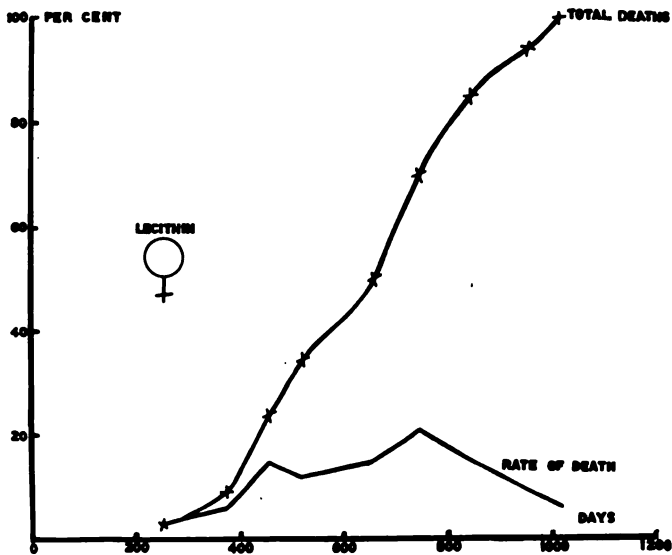


FIG. 8. Mortality curves for lecithin-fed female white mice.

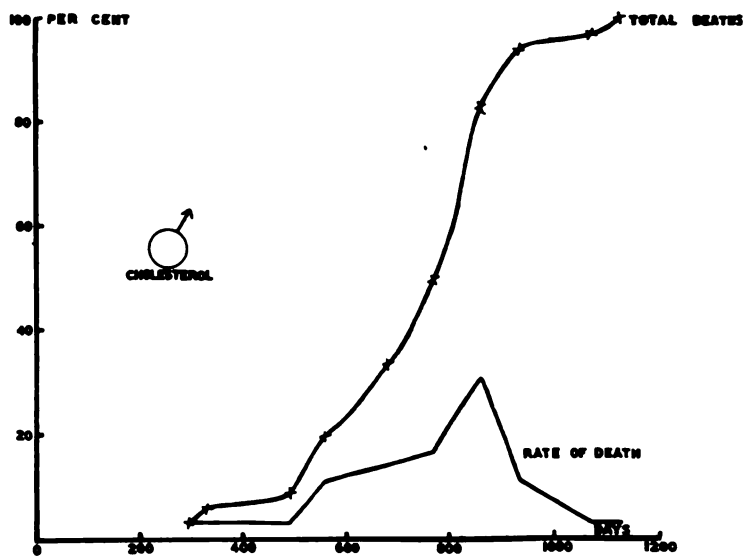


FIG. 9. Mortality curves for cholesterol-fed male white mice.

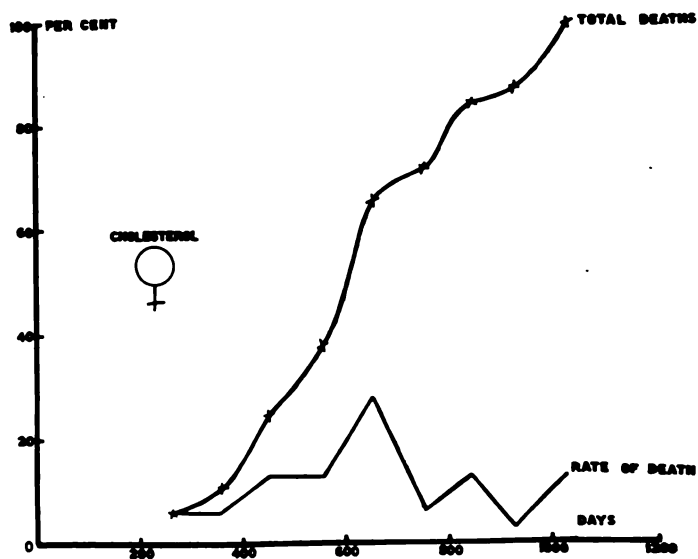


FIG. 10. Mortality curves for cholesterol-fed female white mice.

EXPERIMENTAL STUDIES ON GROWTH.

XIII. LESIONS EXHIBITED BY NORMAL, PITUITARY-, LECITHIN-, CHOLESTEROL-, AND TETHELIN-FED WHITE MICE AT THE OCCURRENCE OF NATURAL DEATH, WITH ESPECIAL REFERENCE TO THE INCIDENCE AND DEVELOPMENT OF SPONTANEOUS CANCER.

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Procedure.

The animals employed in the investigations described in the preceding articles of this series (Papers I to VI and X to XII) were invariably examined for lesions after the occurrence of natural death, except in a small proportion of cases in which delayed detection of death led to decomposition which rendered examination of the tissues useless or impossible.

Gross lesions or tumors manifest to the naked eye on examination of the organs were noted and sections of formaldehyde-hardened material were prepared and examined microscopically. No attempt was made to examine sections from a variety of organs and tissues as a routine procedure, because our facilities and the time available did not permit us to undertake such a task. For this reason a considerable proportion of incipient cases of nephritis, for example, as well as other lesions of interest and importance may have been overlooked. The carcasses of all the animals have been preserved in formaldehyde for further pathological studies. We feel confident that nearly all cases of carcinoma and other new growths were detected and identified except in the small proportion of cases in which postmortem examination of the tissues was impossible.

In a certain proportion of cases the naked eye examination of the tissues revealed no obvious lesions or cause of death. Such cases frequently occurred in very old, senescent animals, in which death was probably due to simultaneous atrophic degeneration of a number of organs.

The results of our postmortem examinations are summarized in Tables I and II and are given in detail in Tables III to XII. No deaths prior to 210 days are included in the tables, such deaths, as explained in the preceding article, being regarded as essentially accidental in origin.

Infections.

Local infections occurred in and were responsible for the death of a relatively large proportion of the males. This is attributable to injuries received in fighting, especially in the neighborhood of the penis and testicles. Late in life, and frequently after complete healing of the traumatisms as far as could be judged externally, these lesions became the center of localized infections, which frequently terminated life by blocking the passage of the urethra.

General infections (pneumonia and β -paratyphoid) were rare, since especial precautions were taken to prevent infection from one animal or cage to another. Among all the males an average of slightly over 4 per cent died, usually late in life, from generalized infections, and among the females less than 1 per cent.

Carcinoma.

Tumors accounted for the death of a great proportion of the animals of both sexes and of all dietary classes. Carcinomas were much more abundant in the females than in the males, occurring in 27 per cent of all the males and 39 per cent of all the females. This figure corresponds closely with the average incidence found by Lathrop and Loeb in a variety of different strains of mice.¹

No definite effect of the dietary administrations upon the frequency of incidence of carcinoma was observed. This fact is significant when the markedly accelerative action of cholesterol

¹ Lathrop, A. E. C., and Loeb, L., *J. Cancer Research*, 1916, i, 1.

TABLE I.

Average Age at Death in Animals with the Following Lesions.

Class of animals.	Carcinoma.	Sarcoma.	Degenerative lesions.	Average duration of life.
Males.				
	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
Normal.....	826	717	998	767
Pituitary.....	823	765	855	792
Lecithin.....	743	681	874	731
Cholesterol.....	848	586	837	764
Tethelin.....	958	766	1,065	866
Females.				
Normal.....	721	746	—	719
Pituitary.....	689	743	—	704
Lecithin.....	674	725	634	677
Cholesterol.....	590	506	871	658
Tethelin.....	970	774	—	800

TABLE II.

Percentage of Animals Exhibiting Certain Lesions at Death.

Class of animals.	Local infection.	General infection.	Carcinoma.	Sarcoma.	Degenerative lesions.	No lesions ascertained.
Males.						
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Normal.....	28	3	25	19	9	16
Pituitary.....	26	3	29	10	9	23
Lecithin.....	9	9	22	19	12	28
Cholesterol.....	3	3	26	3	29	37
Tethelin.....	4	4	35	26	4	26
Females.						
Normal.....	3	0	37	47	0	13
Pituitary.....	3	3	51	20	0	23
Lecithin.....	3	0	32	26	18	21
Cholesterol.....	3	0	50	12.5	21	12.5
Tethelin.....	0	0	25	44	0	31

and tethelin upon the growth of inoculated carcinomas is recalled,² and it demonstrates decisively the truth of the generalization to which other investigators have been led,³ that the conditions favorable to the incidence of carcinoma are quite distinct from those which are favorable to its growth.

No definite effects were observed upon the duration of life of the animals which exhibited carcinomatous neoplasms at death in any of the dietary classes except those which received tethelin (4 mg. daily). In these the prolongation of life of the animals exhibiting carcinomas at death was even more marked than the prolongation of life of the average tethelin-fed animals (132 days in the males, and 249 days, or 33 per cent of the whole normal duration of life, in the females). Since no other classes exhibited the slightest tendency to prolongation of life of the carcinomatous individuals, we must regard this effect as distinctively attributable to the administration of tethelin.

Since carcinoma is essentially a disease which accompanies a measure of senescence, we may infer that the delay in the death of the carcinomatous animals was due to the fact that the onset of senescence in the tethelin-fed animals is markedly deferred, and the spontaneous origin of carcinoma suffered even more than proportionate delay. The fact that the delay in the incidence of carcinoma was *more* than proportionate to the prolongation of the average life may be correlated with the fact that senescent loss of weight, when it does initiate in tethelin-fed animals, is less rapid than it is in normal animals (cf. Figs. 3 and 4 in Paper XI).

Lathrop and Loeb, in their investigations upon tumor incidence and tumor age in mice¹ do not report the death of any animals having tumors at ages exceeding 2 years, and our observations upon mice of all dietary classes, other than the tethelin-fed groups, support the conclusion that the oldest animals do not develop carcinoma. In the tethelin-fed groups, however, the reverse was true, and the carcinomatous growths were encountered most abundantly in the animals which survived the longest.

² Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344; 1915, xxi, 280; 1916, xxiii, 631. Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 309.

³ Woglom, W. H., *J. Exp. Med.*, 1915, xxii, 766. Robertson, T. B., and Burnett, T. C., *J. Cancer Research*, 1918, iii, 75.

The result could not have been influenced by hereditary factors, such as the accidental selection of animals descended from a small number of common ancestors transmitting these peculiarities to their offspring, for the male and female groups of tethelin-fed animals were born in August and October, 1914, respectively, while the cholesterol-fed groups which were selected from the same stock and did not display this peculiarity were born in September and October, 1914; that is, subsequent to the birth of the tethelin-fed males and prior to the birth of the females.

Even more marked than the delay in the *incidence* of carcinoma, was the delay of its *growth* in the tethelin-fed animals. A majority of the animals exhibiting carcinoma in other classes died as a result of growth of the tumor, and consequent interference with the functions of the organs in its vicinity. In the tethelin-fed groups of animals, on the contrary, death in nearly all cases occurred from other and undefined causes, possibly diffuse senescent atrophy of one or more organs, not evident to the naked eye, and the carcinoma was confined to one or more minute spots of from 1 to 6 mm. diameter in the lungs.

This result was very unexpected, since in animals which are afflicted with *inoculated* carcinoma, the administration of tethelin causes marked acceleration of the growth. A reason for this discrepancy may possibly reside in the differing locality of primary incidence in tethelin-fed as compared with the other classes of animals. In all the other classes the primary growth generally occurred in the axilla or in the groin. In the tethelin-fed animals the primary growth did not, as a rule, occur in these localities, or at all events did not make itself grossly manifest there, the only growth, in the majority of cases, being found in the lungs.

Sarcoma.

Several types of sarcoma were encountered, but the most common was a lymphosarcoma, probably identical with the lymphadenoma of mice described by Haaland⁴ and by Murray.⁵

⁴ Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165.

⁵ Murray, J. A., Third scientific report of the investigations of the Imperial Cancer Research Fund. London, 1908, p. 69.

It appears, however, to be more abundant in our stock than in the strains of mice examined by Murray, who reports only four cases in a large number of animals.

This tumor consisted of masses of lymphoid cells widely distributed in the body and sometimes accompanied by lymphoid infiltration of the organs, resembling Hodgkin's disease. In one instance masses of lymphoid tissue were found even in the brain. The distribution of this tumor differed widely from that of carcinoma, and diagnosis, although always confirmed by the microscope, could confidently be made with the naked eye. The lymphoid masses occurred chiefly in the mesenteric glands and the glands adjacent to the ureters and the kidneys, while the thymus was almost invariably the site of a larger or smaller growth and the great majority of animals displayed a marked splenomegaly. The carcinomas, on the contrary, occurred in the subepithelial regions in the groin and axilla and metastasized into the lungs, the thymus remaining unaffected.

The frequency of the incidence of sarcoma was decidedly reduced by the administration of cholesterol; it was one-sixth of the average in all other classes of males and one-third of the average in all other classes of females. On the other hand, the frequency of the incidence of sarcomas (almost all lymphosarcomas) was greater in the tethelin-fed animals, being twice the average of all other classes in both males and females. In the females, however, the incidence did not exceed that found in the normal females. Probably this slightly increased frequency of incidence of sarcomas in the tethelin-fed animals was due merely to the delay in the incidence of carcinoma, permitting a greater proportion of sarcomas to develop.

The duration of life of the small proportion of cholesterol-fed animals which developed sarcomas was decidedly reduced, 131 days below the average duration of life of the normals in the males and 240 days in the females. In other words, sarcoma developed early or not at all. The duration of life of the tethelin-fed animals which developed sarcomas was slightly above normal.

In all groups of male animals the duration of life of those which developed sarcoma was less than that of the animals which developed carcinoma. In each of the groups of female animals, with the exception of those receiving cholesterol or tethelin, the

reverse was the case. In all classes of male animals the average duration of life of the animals which developed sarcoma was less than the average duration of life for all the male animals in the class. In each of the classes of female animals, with the exception of the cholesterol- and tethelin-fed groups, the reverse was the case. There is therefore a correlation between sex and the relative and absolute ages of incidence of these two types of tumors, and the exception afforded by the cholesterol- and tethelin-fed groups may be correlated with the abnormal time relations of growth in these two groups, which resembled one another, but differed markedly from those displayed by the remaining classes of animals (cf. Paper XI).

In eight of the ten groups of animals studied, carcinomas were more numerous than sarcomas.

Degenerative Lesions.

Degenerative lesions, usually of the kidney, but occasionally of the liver or other organs, were frequent in the animals of advanced age, and more especially in the animals which received cholesterol. The unusual frequency of degenerative lesions in the cholesterol-fed animals must be attributed to the extensive deposits of cholesterol which form in various tissues of animals fed unusual quantities of cholesterol,⁶ and which give rise to lesions which subsequently become centers of degenerative changes. The presence of such deposits in the livers and adrenals of our animals was confirmed by Dr. C. H. Bailey, whom we wish to thank for examining a number of specimens.⁷

Degenerative lesions in the kidneys (interstitial nephritis) were most abundant in the cholesterol-fed animals, the percent-

⁶ Chalataw, S. S., *Virchows Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85. Anitschkow, N., *ibid.*, 1913, lvi, 379; 1914, lvii, 201. Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 68; 1915-16, xiii, 60.

⁷ It is of interest to note that Chalataw and Anitschkow failed to elicit the formation of deposits of cholesterol in the liver by administering cholesterol by mouth to rats, although they readily obtained them in rabbits. This may possibly indicate a difference in the lipid metabolism of rats and mice, or more probably may merely be traceable to differences in the dosages administered.

age of observed cases being four times the average for all other classes in the cholesterol-fed males and six times the average for all other classes in the cholesterol-fed females. This fact is remarkable because, according to the authors cited, manifest deposits of cholesterol do not occur in the kidneys of normal animals receiving cholesterol by mouth.⁶ It has, however, been pointed out by Weltmann and Biach⁷ that when the kidneys have been injured, as, for example, in uranium nephritis, cholesterol is deposited in the injured cells and may doubtless cause exaggeration of the injury. We can understand, therefore, how administration of cholesterol might increase and make prominent lesions which would otherwise have remained latent or subacute until death.

Facilities did not permit a special investigation of the question of cholesterol deposits in the kidneys, and furthermore administration of cholesterol, through lack of a sufficient supply, was discontinued a year before the majority of the most marked cases of nephritis developed. By this time the original deposits of cholesterol might be expected to have disappeared, although the lesions caused by them would presumably have remained.

TABLE III.
Normal Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	720	28
" general.....	720	3
Carcinoma.....	826	25
Sarcoma.....	717	19
Degenerative lesions: kidneys.....	998	9
No cause ascertained.....	689	16
Average duration of life.....	767	

⁶ Anitschkow, N., *Deutsch. med. Woch.*, 1913, xxxix, 741.

⁷ Weltmann, O., and Biach, P., *Z. exp. Path. u. Therap.*, 1913, xiv, 367.

TABLE IV.
Normal Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	1,065	3
" general.....	—	0
Carcinoma.....	721	37
Sarcoma.....	746	47
Degenerative lesions.....	—	0
No cause ascertained.....	524	13
Average duration of life.....	719	

TABLE V.
Pituitary-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	515	26
" general.....	1,032	3
Carcinoma.....	823	29
Sarcoma.....	765	10
Degenerative lesions		
Kidneys.....	835	6
Liver.....	895	3
No cause ascertained.....	875	23
Average duration of life.....	792	

TABLE VI.
Pituitary-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	477	3
" general.....	898	3
Carcinoma.....	689	51
Sarcoma.....	743	20
Degenerative lesions.....	—	0
No cause ascertained.....	709	23
Average duration of life.....	704	

TABLE VII.
Lecithin-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	515	9
" general.....	564	9
Carcinoma.....	743	22
Sarcoma.....	681	19
Degenerative lesions		
Kidneys.....	1,031	9
Stomach.....	402	3
No cause ascertained.....	807	28
Average duration of life.....	731	

TABLE VIII.
Lecithin-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	729	3
" general.....	—	0
Carcinoma.....	674	32
Sarcoma.....	725	26
Degenerative lesions		
Kidneys.....	637	6
Ulcers.....	523	9
Intestine.....	961	3
No cause ascertained.....	650	21
Average duration of life.....	677	

TABLE IX.
Cholesterol-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	816	3
" general.....	329	3
Carcinoma.....	848	26
Sarcoma.....	586	3
Degenerative lesions		
Kidneys.....	811	26
Liver.....	1,077	3
No cause ascertained.....	692	37
Average duration of life.....	764	

TABLE X.
Cholesterol-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	925	3
" general.....	—	0
Carcinoma.....	590	50
Sarcoma.....	506	12.5
Degenerative lesions		
Kidneys.....	834	9
Ovary.....	846	9
Liver.....	1,057	3
No cause ascertained.....	641	12.5
Average duration of life.....	658	

TABLE XI.
Tethelin-Fed Males.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	815	4
" general.....	544	4
Carcinoma.....	958	35
Sarcoma.....	766	26
Degenerative lesions, kidneys.....	1,065	4
No cause ascertained.....	871	26
Average duration of life.....	866	

TABLE XII.
Tethelin-Fed Females.

Cause of death.	Average age at death.	Proportion of total deaths.
	<i>days</i>	<i>per cent</i>
Infections, local.....	—	0
" general.....	—	0
Carcinoma.....	970	25
Sarcoma.....	774	44
Degenerative lesions.....	—	0
No cause ascertained.....	703	31
Average duration of life.....	800	

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EXPERIMENTAL STUDIES ON GROWTH.

XIV. FURTHER EXPERIMENTS ON THE INFLUENCE OF TETHELIN UPON THE GROWTH OF THE WHITE MOUSE.

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(Received for publication, January 3, 1919.)

In 1914 two experiments on the influence of tethelin upon the growth of the white mouse were begun. In one of them, consisting of twenty-four animals, the administration (4 mg. per day) was continuous, while in the other, consisting of sixteen animals, the administrations were discontinuous, being confined to three periods of 4 weeks each in the first 30 weeks of life. The results of these experiments, up to the 60th week of life, were reported in 1916 in Paper IV of this series,¹ while the results from the initiation of the administrations until the natural death of the animals are reported in Paper XI.

Further experiments were not conducted at that time, owing to lack of facilities for the production of sufficient tethelin for daily administration to so many animals. In 1916 more abundant supplies of tethelin became available and a third and fourth group of animals were started upon tethelin, and in 1917 a fifth, comprising a total of 134 animals in five different groups: three of females (total, 88 animals) and two of males (total, 46 animals).

The effectiveness of discontinuous administrations of tethelin in producing the characteristic deformations of the growth curve that accompany the administration of tethelin, led to the investigation of the effects of a single relatively brief period of administration in two of the three new experimental groups; namely, in a group of 24 females started in 1916, and a group of 22 males started in 1917. In these experiments tethelin (4 mg. per day) was administered for 8 weeks only, from the 4th to the 12th

¹ Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397.

week of age, and then discontinued for the remainder of the animal's life. In the remaining experiment 48 females received tethelin continuously (4 mg. per day).

The results are shown in Tables I to III and Figs. 1 to 3. The figures show in every case the characteristic deformation of the growth curve, resulting in a marked concavity in what would normally be the most rapidly rising part of the curve. In no other experimental group except those receiving cholesterol, and in none of the six groups of normals studied during this period (cf. Paper X) has this marked concavity in the curve of growth been noted, and it has occurred in five separate groups of tethelin-fed animals.

The concavity in the curve of growth is the expression of preliminary retardation preceding sexual maturity, followed by a secondary or compensatory acceleration accompanying and succeeding sexual maturity.

In our previous reports we inclined to the view that the acceleration, in common with the retardation, was directly due to tethelin. We now believe that while the retardation is due to tethelin, the acceleration is due to compensatory factors which develop in the animal itself in response to the abnormal dosage of the active principle of the anterior lobe of the pituitary body. We prefer this view since the compensatory acceleration is more intense when the retarding factor, tethelin, is discontinued after the 12th week of age and 8th week of the administration. This fact is evident from Figs. 2 and 3.

In adult animals which receive tethelin continuously, the compensatory acceleration disappears, and the curves of the tethelin-fed and normal animals become nearly parallel, the normals increasing in weight, in fact, more rapidly than the tethelin-fed animals until the onset of senescence. But in animals which have only received tethelin from the 4th to the 12th week the acceleration continues in most marked degree. The effects of brief administration upon late growth will be reported subsequently but it may be stated that by this means we have succeeded in producing a number of exceptionally large animals.

In the females of 1916, receiving the brief administration of tethelin, the secondary acceleration led very early (at 11 weeks) to intersection of the normal curve by the curve of the tethelin-

fed animals, retardation being displayed only by the acceleration which succeeded it. In the males of 1917 the intersection with the curve of the 1916 normals² occurred at a later period (18 weeks). The growth curves of animals receiving tethelin continuously do not intersect the normal curve until advanced old age, when the delay of senescence in the tethelin-fed animals confers an advantage upon them relatively to the normals.

Since the direct action of tethelin would thus appear to consist exclusively, so far as the whole animal is concerned, of retardation of growth, the question presents itself how the acceleration of growth of epithelial tissues *in situ* arises which is noted in the effects of tethelin upon the healing of superficial wounds³ and upon the growth of inoculated carcinoma.⁴ Two possibilities suggest themselves; namely, that tethelin accelerates the growth of epithelial tissues and directly or indirectly retards the growth of the other and collectively more bulky tissues, or that tethelin retards the growth of all tissues, but indirectly accelerates the growth of epithelial tissues *in situ* by disproportionately retarding the growth of other tissues, and thus favoring the epithelial tissues in the competition for nutritive materials. Between these two alternatives a decision may be reached only by experiments *in vitro*, for which the opportunity has hitherto been lacking.

² No 1917 normals were available for comparison, but since in 1916 our stock was already approaching a stable type (cf. Paper X) it is probable that the 1917 normals would not have differed appreciably from those of 1916.

³ Robertson, T. B., *J. Am. Med. Assn.*, 1916, lxvi, 1009. Barney, E. L., *J. Lab. and Clin. Med.*, 1917-18, iii, 480.

⁴ Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1916, xxiii, 631.

TABLE I.

Tethelin-Fed Females, 1916. Continuous Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	30	10.23	-2.20	5.8	19.6
5	48	11.78	-1.89	5.4	22.0
6	48	13.26	-1.82	4.8	19.4
7	48	14.65	-1.33	3.8	18.4
8	48	15.85	-1.09	3.2	17.3
9	48	16.59	-0.91	2.8	14.5
10	48	17.03	-0.70	2.4	13.5
11	48	17.36	-0.83	2.4	14.2
12	48	17.70	-0.72	2.1	13.8
13	48	18.07	-0.49	1.4	14.1
14	48	18.31	-0.92	2.4	14.6
15	48	18.67	-0.75	2.0	15.6
16	48	18.91	-0.99	2.7	14.9
17	48	19.22	-0.97	2.5	15.1
18	48	19.21	-0.85	2.1	15.4
19	48	19.35	-0.96	2.4	15.3
20	48	19.66	-1.22	3.0	15.4
21	48	19.93	-0.92	2.2	15.2
22	48	19.86	-1.03	2.5	14.8
23	48	20.13	-1.09	2.5	14.8
24	48	20.16	-1.23	2.9	14.9
25	48	20.39	-1.20	2.5	15.1
26	48	20.54	-1.22	2.5	13.9
27	48	20.56	-1.44	3.0	13.7
28	48	20.58	-2.13	4.4	14.0
29	46	20.44	-2.33	4.5	14.8
30	45	20.76	-2.07	4.2	14.7
32	48	21.38	-1.73	3.5	14.0
34	48	21.81	-1.36	2.8	14.4
36	48	22.10	-1.55	3.1	15.3
38	48	22.31	-1.59	2.9	15.3
40	48	22.67	-1.26	2.2	16.6
42	47	23.09	-1.39	2.4	14.3
44	47	23.78	-0.22	0.4	13.8
46	47	23.55	-0.69	1.4	14.3
48	47	24.04	-0.03	0.1	15.3
50	47	24.20	+0.16	0.3	15.2

TABLE II.
Tethelin-Fed Females, 1916. Brief Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1916.	Times probable error of difference.	Variability.
<i>wks.</i>		<i>gm.</i>			<i>per cent</i>
4	13	10.54	-1.89	5.6	9.0
5	24	12.63	-1.04	2.4	20.0
6	24	13.92	-1.16	2.7	16.2
7	24	15.54	-0.44	1.0	15.9
8	24	16.46	-0.48	1.2	15.0
9	24	17.23	-0.27	0.6	14.8
10	24	17.58	-0.15	0.4	14.2
11	24	18.35	+0.16	0.4	13.4
12	23	19.46	+1.04	2.4	13.6
13	23	19.91	+1.35	3.4	11.8
14	23	20.43	+1.20	2.7	13.3
15	23	20.63	+1.21	2.7	12.6
16	23	21.04	+1.14	2.6	11.7
17	23	21.46	+1.27	2.8	11.4
18	23	21.98	+1.92	4.3	12.0
19	23	22.48	+2.17	4.6	12.1
20	23	22.20	+1.32	2.9	11.9
21	23	22.70	+1.85	3.7	12.7
22	23	22.67	+1.78	3.6	12.4
23	23	22.80	+1.58	3.2	12.1
24	23	23.22	+1.83	3.7	12.3
25	23	23.35	+1.76	3.1	13.0
26	23	23.65	+1.99	3.4	12.6
27	23	23.52	+1.52	2.5	14.3
28	23	23.59	+0.88	1.4	15.1
29	23	23.52	+0.75	1.2	13.3
30	23	24.00	+1.17	1.9	14.7

TABLE III
Tethelin-Fed Males, 1917. Brief Administration.

Age.	No. weighed.	Weight.	Deviation from normal of 1914.	Times probable error of difference.	Variability
<i>lbs.</i>		<i>gm.</i>			<i>per cent</i>
4	14	10.50	-1.79	3.4	18.5
5	22	12.05	-1.20	2.3	21.0
6	22	13.82	-1.22	2.4	19.6
7	22	15.16	-1.67	3.4	17.0
8	22	15.57	-2.51	5.2	15.9
9	22	16.95	-2.05	4.3	14.3
10	22	17.86	-1.77	3.9	13.0
11	22	18.57	-1.39	3.2	11.0
12	22	19.75	-0.60	1.5	10.9
13	22	19.66	-1.26	2.8	9.5
14	22	20.84	-0.47	1.2	7.4
15	22	21.41	-0.42	1.0	5.3
16	22	21.84	-0.31	0.7	7.7
17	22	22.36	-0.06	0.1	6.8
18	20	23.18	+0.60	1.4	6.8
19	20	23.58	+0.87	2.1	7.1
20	20	24.08	+1.06	2.5	7.3
21	20	24.45	+1.35	3.2	7.5
22	20	24.68	+1.26	2.9	7.7
23	20	25.05	+1.56	3.5	7.9
24	20	25.43	+1.80	4.5	7.1
25	20	25.80	+1.95	4.6	8.1
26	20	25.80	+1.70	4.3	6.5
27	20	25.90	+1.59	3.9	7.6
28	20	25.95	+1.64	3.7	7.9
29	19	25.97	+1.32	3.3	6.9
30	20	26.65	+1.74	3.7	7.8

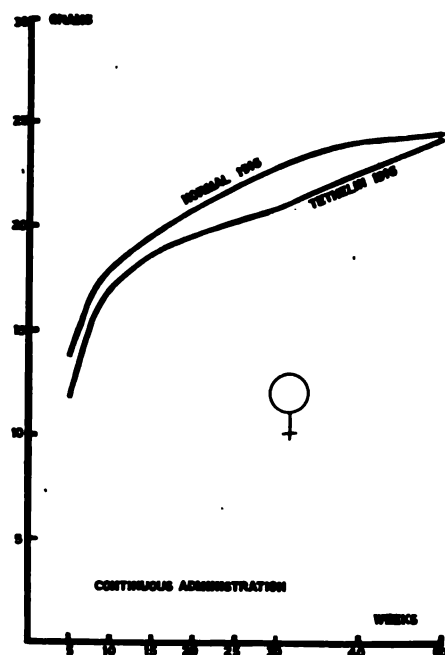


FIG. 1. Influence of tethelin upon the growth of female white mice. Administration continuous. Dosage 4 mg. per day.

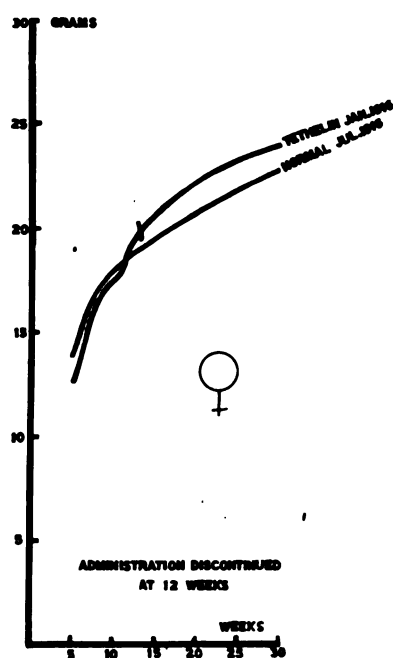


FIG. 2. Influence of tethelin upon the growth of female white mice. Administration discontinued at 12 weeks (indicated by vertical cross mark). Dosage 4 mg per day.

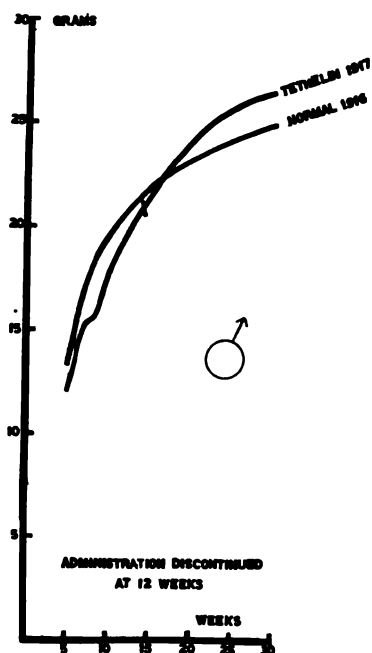


FIG. 3. Influence of tethelin upon the growth of male white mice. Administration discontinued at 12 weeks (indicated by vertical cross mark.) Dosage 4 mg. per day.

ABSORPTION SPECTRA OF ACID HEMATIN, OXYHEMOGLOBIN, AND CARBON MONOXIDE HEMOGLOBIN. A NEW HEMOGLOBINOMETER.

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(Received for publication, January 24, 1919.)

This paper contains spectrophotometric data on two hemoglobin derivatives. The transmissions of solutions of oxyhemoglobin, acid hematin, and carbon monoxide hemoglobin for light in the visible and ultra-violet portions of the spectrum are given. The data have a certain abstract interest. They serve to correlate properly spectrophotometric values with oxygen capacity. The immediate purpose in securing them was that they might serve as a basis for work on colorimetric hemometers, a problem which is discussed at the end of this paper. The optical properties of a new hemoglobinometer are described.

HISTORICAL.

The visible bands of oxyhemoglobin first described by Hoppe-Seyler (1) have received considerable attention, more particularly as regards their position. Soret (2) was the first to recognize the great ultra-violet band of oxyhemoglobin, a band which he observed with a fluorescing eyepiece. Later Gamgee (3) using a quartz spectrograph and the sun as a source, made a more extensive investigation of the ultra-violet absorption spectra. He showed that the great ultra-violet band was common to oxyhemoglobin, reduced hemoglobin, CO- and NO-hemoglobin, hemochromogen, hematin hydrochloride, methemoglobin, and hematoporphyrin. Gamgee did not find such a band in the spectra of bilirubin, hydrobilirubin, or urobilin. A paper by Rost, Franz, and Heise (4) contains excellent plates of the spectra of many of the hemoglobin derivatives, together with an extensive summary of earlier work. Lewin, Miethe, and Stenger (5) have also published extensive qualitative spectrographic data of this sort.

Rollett (6) gives a picture, only roughly correct, showing the change in width of the absorption bands of oxyhemoglobin with dilution. Vier-

ordt (7) was the first to study the quantitative absorption of these compounds throughout the visible spectrum. His tables and curves were made on a very early type of spectrophotometer having large instrumental errors. Cherbuliez (8), in 1890, published certain spectrophotometric data on the hemoglobins which he had made with an improved instrument. His absorption curves given for the more central portions of the visible spectrum are more nearly correct. The α -band of oxyhemoglobin is given as shallower than the β -band, an error difficult to avoid because of the disturbing effect of the very high transmission to the red side of the α -band.

Aside from these two determinations of absorption curves there have been many determinations of certain absorption constants and relations, in part undertaken with the view of fixing a spectrophotometric method for the quantitative determination of oxyhemoglobin and other hemoglobins.

If T is the fraction of light of a given wave-length transmitted by a substance, then the equation $T = 10^{-e}$ defines a number e known as an extinction coefficient. The extinction coefficient is the cologarithm of the transmission. It was first shown by Beer (9) that for a solution a simple absorption relation exists between the concentration, c , the thickness, t , and the extinction coefficient,

e ; namely, $\frac{ct}{e} = \text{constant}$. This equation holds under a wide range

of conditions. For oxyhemoglobin, carbon monoxide hemoglobin, and acid hematin it would seem at least to hold within the range of dilution here used (whole blood 1:100 to 1:2,000). Butterfield (10) has shown that the relation holds in the case of oxyhemoglobin for a range of dilution of whole blood 1:1 to 1:160. Reid (11) has shown that oxyhemoglobin forms a true solution.

While e is understood to be the extinction coefficient for a single wave-length, practically it is the mean e for a sufficiently small spectral interval, the interval given by the exit slit of the spectrophotometer. The value of the constant of the equation being determined at such an interval for a known concentration, c , this value furnishes a basis for the calculation of any c by the instrumental determination of the e . By properly choosing the spectral interval and using concentrations giving extinction coefficients within a certain optimal range, the determination may be made to within 1 per cent. With uniformity in slit widths such determinations are absolute, variations between different in-

struments being only within observational errors of the individual instrument.

With the thickness of solution equal to 1 cm. it has become customary to designate the constants by the letter A . The ratio of two such constants determined for two separate spectral intervals is a ratio independent of the concentration and a characteristic of the substance.

$$\frac{A'}{A} = \frac{c'/e'}{c/e} = \frac{e}{e'}$$

The principal determination of the values of these constants A_0 and A'_0 for oxyhemoglobin, are as follows:

Hüfner (12, 13)

$A_0 = 2.070$ (5540 to 5650 Ångström units)¹

$A'_0 = 1.312$ (5315 " 5425 " ")

$\frac{A_0}{A'_0} = 1.578.$

¹ The Ångström unit is one ten-millionth of a mm. or one ten-thousandth μ . The Fraunhofer lines often referred to as reference lines have the wave-lengths in Ångström units given below. The values due to Rowland are corrected on the basis of the accepted values for the primary standard cadmium line and the secondary standard iron arc lines of Fabry-Buisson (see Fowle, F. E., *Smithsonian Miscellaneous Coll.*, Publication 2269, 1918, lxiii, No. 6):

	Å. u.		Å. u.		Å. u.		Å. u.		Å. u.		Å. u.
B	6867.2	E ₂	5269.55	F	4861.35	L	3820.44	Q	3286.76	T	3020.65
C	6562.8	b ₁	5183.62	G	4307.92 4307.75	M	3727.64	R	3181.26 3179.33	t	2994.42
D ₁	5895.95	b ₂	5172.69	h	4101.85	N	3581.19	S ₁ S ₂	3100.67 3100.31 3099.93	U	2947.89
D ₂	5889.98	b ₃	5169.05 5168.90	H	3968.48	O	3441.00 3440.61				
E ₁	5270.39 5270.27	b ₄	5167.51 5167.33	K	3933.68	P	3361.18	s	3047.61		

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Cherbuliez (8)

$$A_c = 2.038 \text{ (5530 to 5580 Ångström units)}$$

$$A_o = 1.326 \text{ (5360 " 5410 " ")}$$

$$\frac{A_o}{A'_o} = 1.537$$

De Saint Martin (14) (polarization spectrophotometer)

$$A_o = 2.153 \text{ (5572 to 5683 Ångström units)}$$

$$A'_o = 1.330 \text{ (5380 " 5490 " ")}$$

$$\frac{A_o}{A'_o} = 1.621$$

Bardachzi (15) (polarization spectrophotometer)

$$A_o = 2.094 \text{ (5540 to 5650 Ångström units)}$$

$$A'_o = 1.334 \text{ (5315 " 5425 " ")}$$

$$\frac{A_o}{A'_o} = 1.56$$

Butterfield (16) (polarization spectrophotometer)

$$A_o = 1.87 \text{ (5561 to 5646 Ångström units)}$$

$$A'_o = 1.18 \text{ (5335 " 5420 " ")}$$

$$\frac{A_o}{A'_o} = 1.58$$

Butterfield (17)

$$\alpha_1 = 18.42 \text{ (green mercury line, 5459 Ångström units)}$$

$$\alpha_2 = 21.00 \text{ (yellow " lines, 5770 and 5790 Ångström units)}$$

(α represents the Napierian cologarithm of the transmission of a 1 per cent solution.)

Butterfield's figures for A differ from those of other reliable observers by approximately 11 per cent. In determining the transmissions by oxyhemoglobin of the green mercury line or the two very adjacent and equal yellow mercury lines many of the technical difficulties connected with accurate spectrophotometric measurements disappear. Care no longer needs to be exercised in making wave-length measurements; there is much more latitude in the choice of slit width relations and various reflection errors are removed.

The α_1 and α_2 given by Butterfield might be transformed to correspond to the notation otherwise used:

$$A_o^{5459} = 1.25$$

$$A_o^{577, 579} = 1.096$$

EXPERIMENTAL.

Apparatus.

The apparatus used was a Hilger sector photometer and quartz spectrograph, size C, giving a spectrum 18 cm. long, extending from 2100 to 7000 Ångström units. The light from a single source passed by means of appropriate quartz prisms along two separate and symmetrical paths, one above the other, in each of which was placed a so called rotating sector consisting of a circular disk with two symmetrical open sectors. The openings of the lower rotating sector were fixed in size and each was a quadrant. The openings in the other could be varied from 90–0°. To the sector photometer was bolted the absorption cell so that it could be easily demounted for filling and returned to exact alignment. The double absorption cell was made from a single piece of plate glass by drilling it with two holes slightly larger than the two beams of light and exactly centered on them. Through the edge of the glass plate entrance holes were drilled into the first ones. Windows of plane clear quartz were then fastened on each face of the glass plate with DeKhotinsky cement, thus making two cells each 1 cm. thick.

This construction gave a rigid alignment of the cells in symmetrical position with respect to the light paths. In the upper cell was placed distilled water and in the lower the absorption fluid. The absorption of the cell itself was thus corrected for at first hand. Opposite the lower cell was the fixed opening sector, and opposite the upper cell the variable sector.

The light coming along the two paths fell in juxtaposition on the collimator slit of the spectrograph and passed parallel in two beams to be dispersed into two spectra, one directly above the other, and so photographed on the plate. By varying the opening of the one sector the exposure of this comparison beam was cut down one step at a time. Twenty-four such simultaneous pairs of spectra could be taken on one plate. The wave-length at which a pair of spectra matched in density was the wave-length having an extinction coefficient corresponding to that sector opening. In a paper by Howe (18), and in one by myself (19), it has been quite conclusively shown that the extinction co-

efficient is the logarithm of the ratio of the fixed to the variable opening.

The plates used were the Wratten and Wainwright panchromatic of London, and of Eastman manufacture. They were given tank development according to the formulas accompanying them. The iron spark, or for the mid-visible spectrum sometimes the D lines of the sodium flame were always photographed on the plate as a comparison scale and used in connection with a wave-length scale furnished for the instrument by Hilger. The scale as checked against the spectra of several metals had errors less than the width of the smallest interval in which the two spectra could be compared.

The light source for the ultra-violet determinations was a condensed iron spark, and for the visible, a Nernst lamp with the filament vertical. At the beginning and end of each plate exposures were made with both cells filled with water in order to show that the two dispersed beams were of equal intensity, or to determine the relative sector openings required to give such equality. After the instrument was in adjustment a balance, with equal sector openings, could be obtained correct to within an extinction coefficient of $1/100$. To compensate for this error the sector settings required for balanced beams were included on each plate and the extinction coefficients corrected by the addition of this balance constant in each case. The plates often balanced to within a fraction of $1/100$. For the ultra-violet the balance was not determinable with as much precision, the smallest observable correction being about $1/100$. In making exposures it was possible for one sector to present its opening once oftener than the other sector. The sectors made 120 revolutions per minute. By increasing the exposures to 1 minute or more, sometimes to 10 minutes, and through repeated observations, this source of error was largely eliminated. A slit opening of 0.015 mm. was uniformly used. This corresponds with an interval of 3.5 Ångström units on the plate at 5500 Ångström units and of 0.3 Ångström units in the far ultra-violet.

The plates were read in a dimly lighted room by holding them above an illuminated white background and comparing the spectral pairs through a slit in a card, this slit being from 20 to 40 Ångström units wide (referred to 5500 region). In the visible region final readings were usually made through the smaller slit.

In the ultra-violet a larger slit was used sometimes which measured in width from 5 to 20 Ångström units. The wave-length position was read off at the center of the slit from the wave-length scale held in position on the plate at the spectrum in question.

Solutions.

The oxyhemoglobin, carbon monoxide hemoglobin, and acid hematin solutions were prepared by the dilution of human blood, usually my own. The diluting fluids were 0.1 per cent sodium carbonate, freshly prepared, 0.4 per cent ammonia water, and 0.1 N hydrochloric acid. For the preparation of carbon monoxide hemoglobin, the solution in the ammonia flask was immediately saturated with illuminating gas. The blood was drawn with a recalibrated Record syringe from a vein, and equal volumes were added to each of the fluids in the proportion 1:100. The estimation of hemoglobin content of the blood was then made on the material in the hematin flask and further dilution to about 1:200 made accordingly. The estimation of hemoglobin content was made on a colorimeter (20) using an artificial color match for hematin. The error in thus preparing the various solutions used from time to time so as to be of the same relative concentration was not large. It was eventually compensated for. In the case of the visible portion of the oxyhemoglobin absorption this was in part done by reducing each set of data to a uniform level through correcting it to correspond to a certain fixed extinction coefficient at the bottom of the α -band. The data for the visible portion of the acid hematin absorption were all obtained in a short interval of time and the errors as between the different solutions were small.

After the various portions of the absorption curves were determined they were corrected for relative concentrations and checked at various points by using solutions made up from blood of known oxygen capacity. The oxygen capacity of fresh oxalated human blood was determined by the method of Van Slyke (21) and simultaneously a 1:200 dilution made with the various diluting fluids, all glassware being recalibrated. The carbon monoxide absorption curve was wholly determined from two such solutions. It would have been easier and better to have had such material

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for the determination of the other two curves. It was believed that in a complete redetermination an almost prohibitive amount of labor would have to be expended before changes in the curves as already computed would be justified. Certain corrections were however made. The question of absolute concentration of the solutions in gm. per liter will be considered later.

Oxyhemoglobin, when oxygen saturation has been reached, and carbon monoxide hemoglobin, when kept saturated with the gas, form under these circumstances true solutions, which, when kept cold, remain stable for at least a week, and probably for a month or more.

The acid hematin as thus prepared acts for the present purpose as if it formed a true solution. After several months particles settle out which thereafter cannot be brought again into stable suspension. This process is accelerated if the material is kept at room temperature and is most apparent in very dilute solutions. In case the hematin mixture is somewhat more concentrated than $\frac{1}{10}$ blood dilution, some particles settle out almost immediately. Under the above conditions of preparation the hematin solution assumes in an hour a spectrophotometric condition which remains nearly constant for several days and even for several weeks.

Relation of Time to Color Depth of Acid Hematin.

It is known (22) that the hematin solution does not assume its full depth of color immediately. In order to determine just how rapidly it does develop its full color depth we made readings on ten preparations of acid hematin from the bloods of as many individuals. The first set of readings was made during the 3rd minute after the preparation of the hematin, the second set during the 15th minute, another during the 30th minute, and a last set on the following day. As a result of a long previous experience I was able to assume that the solution had reached a stable condition within 24 hours. The most careful spectrophotometric determinations have not shown any demonstrable increase in color depth within the next 10 days. All readings were therefore calculated as percentages of these final values and averaged. It seemed reasonable to assume that the rate of formation of acid

hematin should be a rectangular hyperbola of the general form $xy = -c$, when x measures the time in minutes and y is the per cent by which the acid hematin falls short of its full color depth. Using the value for y corresponding to 30 minutes for x , the constant c of the equation is determined as 40. The values of y for 3 minutes and 15 minutes fit the curve given by the value of c very closely, justifying the assumption that the curve has the form of a hyperbola. Hence the equation for the color density of a newly prepared acid hematin solution is $xy = -40$, where x is the time in minutes and y the percentage short of full color. A graph (Curve D) of this equation is included among the curves of Fig. 2, the squares of the figure being ten units each.

The further trial was made of following a specimen through from 1 to 40 minutes, making readings continuously and averaging them by tens. The points so obtained lay very closely on the curve.

It will be seen that in 20 minutes $y = -2$ and in 40 minutes $y = -1$. In other words, the specimen then has reached 98 and 99 per cent respectively of its full density. It will also be seen that if the specimen is read in 2 minutes the density falls short 20 per cent, or is 80 per cent of the final figure.

The equation not only shows how rapidly the solution reaches its full density, but what correction should be added to a reading when it is made early.

Observations.

The ultra-violet portion of the acid hematin curve was determined from eight plates made on the bloods of four individuals. The sectors were set for variations of tenths in extinction coefficients. The various plates differed only slightly from each other. The data given are a mean of those derived from five plates of my own blood.

For the visible portion of the acid hematin curve seven plates were made using my own blood and with such settings that the extinction coefficient varied by hundredths. Three of the plates were made on the day the solutions were prepared, the others on the 2 following days. The plates were all consistent. They were read, their points plotted, and a mean curve was drawn

through the locus. The discrepancies ran not over 2 per cent on either side of this curve. The plates were again read to eliminate errors of judgment and the mean of the new locus was taken as the true curve, the discrepancies with two or three exceptions having become less than 1 per cent.

Determinations of the ultra-violet absorption of oxyhemoglobin were made on the same bloods as those used for acid hematin, ten plates being made. They showed variations in the region 3200 to 4300 Ångström units, which were not then understood. Some of the solutions showed transmissions in the region 3200 to 3800 Ångström units, which corresponded to extinction coefficients as much as 15 per cent greater than those given. The great ultra-violet band fell short of its full depth by similar amounts. It would seem that these variations were due to incomplete oxygenation of the hemoglobin.

Oxygenation resulting in the dilution of venous blood would require at the most 1 cc. of oxygen per liter of solution. At room temperature, water contains about 6 cc. of dissolved oxygen, and the oxyhemoglobin solution made therefrom would soon come into equilibrium with the atmosphere. In recently distilled water, equilibrium would be less readily established. It is probable that in the discrepancies observed solutions using freshly distilled water were not sufficiently shaken. The effect of blood plasma present in the proportion of about 1:200 will be discussed later.

The ultra-violet portion of the oxyhemoglobin curve as obtained from certain apparently satisfactory plates was further checked on plates made from thoroughly oxygenated blood of known oxygen capacity. The form and depth of the curve at the great ultra-violet band centering on 4140 Ångström units was determined on solutions $\frac{1}{4}$ and $\frac{1}{10}$ as dense as those otherwise used. Here the curve was likewise carefully checked and discrepancies were small when the oxygen saturation was to be considered as satisfactory.

The visible portion of the oxyhemoglobin curve was at first determined from fourteen plates with my own blood. Except for short portions of the steep slopes of the curve the settings for extinction coefficients varied by hundredths. The plates having been correlated on the basis of the readings for the ex-

tion coefficients at the maxima and minima points of the curve, all the plates were then read and their points plotted, the whole was rechecked for errors in judgment, and a curve was drawn through the locus. The discrepancies were somewhat less than those obtained in the visible portion of the acid hematin curve. The small extinction coefficients for the high transmission in the red were determined from the data of six plates, and the results checked by making readings with double the concentration.

The critical points of the curves for oxyhemoglobin and acid hematin, both in the ultra-violet and in the visible, were now checked on solutions of known oxygen capacity. About twenty plates in all were made for this purpose. They served at the same time to give data for the determination of the absolute concentration of the solutions to which the curves corresponded.

Incomplete data for the visible portion of the oxyhemoglobin curve had previously been obtained on a spectrophotometer at the physical laboratory of the United Gas Improvement Company. This was a Hilger quartz monochromatic illuminator coupled with a potassium photoelectric cell and galvanometer. A set of readings obtained with this apparatus for the region 4300 to 6000 Ångström units is consistent with that obtained by the photographic method except that the transmissions at the α - and β -bands, particularly the former, are not so low as they should have been. The dispersion of the instrument was too small for the purpose, and the slit width of 0.075 mm. used was too large. The blue and violet portion of the curve is, however, very closely checked and, as could be expected, the height of the α - β -peak was correctly observed.

I had originally made a determination of the absorption curves of oxyhemoglobin and acid hematin on a visual polarization spectrophotometer at the physical laboratory of the Corning Glass Works. These data were incorrect, the discrepancy being accounted for on the supposition of an error in the zero adjustment of the instrument. In order to check this point Dr. J. T. Littleton, Jr., of that laboratory, has made two redeterminations of the oxyhemoglobin curve on iced material which I sent him. His observations are consistent with my data as they now stand.

The absorption curve for carbon monoxide hemoglobin was obtained from the data of about ten plates made on two solutions

of known oxygen capacity. Only the Nernst lamp was used as a source and the ultra-violet absorption determined out to a little beyond 3100 Ångström units. The depth of the great ultra-violet band was determined on solutions $\frac{1}{10}$ as dense as those otherwise used. It was noticed here too that there were variations in the depth of this band, amounting in a week to a 20 per cent decrease in extinction coefficients for an iced specimen which had been opened several times. The band was brought back to its original depth by again passing illuminating gas through the solution. The height of the peak at 3760 Ångström units decreased likewise very considerably and was as easily restored. Changes in the visible absorption were small if present. The data are derived from freshly gassed solutions.

Concentration of the Solutions.

The concentration of the solutions here used may be calculated from the absorption constants as determined by Hüfner, Cherbuliez, de Saint Martin, Bardachzi, and Butterfield. To do this I calculate from my curve a mean e for the spectrum interval as used by the author in question. The product of this mean e and the author's constant gives a figure for the density of my solution. It happens that the mean e is very nearly equal to the e corresponding to the average transmission for the interval. The data are given in Table I.

If we disregard Butterfield's figures, the concentration would seem to be about 0.85 gm. per liter. As will be seen, however, Butterfield's figures are the correct ones. There is another very direct way of arriving at the concentration of the solutions used, through the determination of the oxygen capacity of the blood used.

Barcroft (23) shows that oxygen combines with hemoglobin in the ratio of one molecule of oxygen to one atom of iron, and further that there is one atom of iron in the hemoglobin molecule. If we accept this proposition, there remains only the question as to what is the percentage of iron in the hemoglobin molecule; what is the molecular weight of hemoglobin? There is very little discrepancy in the figures given by different authors for the iron content of hemoglobin. The accepted figure is about 0.335 per

cent (24). On the basis of this figure the oxygen capacity is 1.34 cc. per gm. of hemoglobin. This figure for oxygen capacity is subject to one correction. It is a maximum figure. Barcroft

TABLE I.

Concentration Data for My Oxyhemoglobin Solutions Based on Regions and Constants of Various Authors.

Author.	Region.	Constant.	Mean σ .	Concentration.	Ratio.	
Hüfner.....	$\alpha\text{-}\beta$	2.070	0.411	0.85	1.54	
“	β	1.312	0.633	0.83		
Cherbuliez.....	$\alpha\text{-}\beta$	2.038	0.418	0.851	1.577	
“	β	1.326	0.659	0.872		
de Saint Martin.....	$\alpha\text{-}\beta$	2.153	0.419	0.90	1.556	
“ “ “	β	1.330	0.651	0.865		
Bardachzi.....	$\alpha\text{-}\beta$	2.094	0.411	0.86	1.54	
“	β	1.334	0.633	0.843		
Butterfield.....	$\alpha\text{-}\beta$	1.87	0.409	0.764	1.58	
“	β	1.18	0.646	0.762		
“	5460	1.25	0.61	0.763		
“	{ 5770	1.096	0.688	0.754		
“	{ 5790					

(23) has shown that the dissociation curve of hemoglobin (the oxygen saturation curve) has the formula

$$\frac{y}{100} = \frac{Kx^n}{1 + Kx^n}$$

where y is the percentage saturation of the hemoglobin with oxygen, x the oxygen pressure in mm. of mercury, n a number 2.5, and K a constant which varies slightly with normal individuals, averaging about 0.0003.

In measuring oxygen capacity the blood was saturated by spreading it thinly over the inner surface of an open, slowly rotating, conical, separatory funnel. The funnel was so rotated for about 10 minutes before pipetting off the specimen to be examined.

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At atmospheric pressure the oxygen pressure is about 180 mm. of mercury. Substituting this figure for x in the formula, y becomes 99.2 per cent. The figure for the cc. of oxygen in 1 gm. of hemoglobin under the above conditions is therefore

$$\frac{99.2}{100} \times 1.34 \text{ cc.} = 1.33 \text{ cc.}$$

Accordingly a 1:201 dilution was made in the various solvents from blood whose oxygen capacity was being determined. The concentration of the solution thus produced when expressed in gm. of oxyhemoglobin per liter was equal to $\frac{10}{201 \times 1.33}$ times the figure for the oxygen capacity of 100 cc. of the whole blood. Extinction coefficients derived from such solutions of known concentration were used to ascertain the concentration of the solutions for which the original absorption curves had been determined. This was accomplished by making use of the fact that the ratio of corresponding extinction coefficients of any two solutions of a substance is equal to the ratio of their concentrations. If the extinction coefficients are known in each case, the concentration of one solution can be deduced from that of the other.

Eight plates were made from four oxyhemoglobin solutions of known concentration. The extinction coefficients at the bottoms of the α - and β -bands as computed from these plates were compared with the figures 0.713 and 0.67 obtained for the corresponding extinction coefficients of the oxyhemoglobin curve. The concentration of this original oxyhemoglobin solution as thus computed at the α - and β -bands was respectively 0.758 and 0.759 gm. per liter. The height of the peak between these two bands as had been computed for Table III was then slightly changed (from 0.408 to 0.406) so as to correspond to a concentration of 0.7585 gm. per liter when similarly referred to the average readings of these eight plates.

If we give these two figures for concentration as derived at the bands an equal weight with the concentration figures of Table I due to Butterfield, we obtain an average of 0.75975 or 0.76 as the gm. per liter of oxyhemoglobin in the solution to which the curve corresponds.

In the case of acid hematin the extinction coefficient of these known solutions at the nearly level interval 5500 to 5580 Ångström units was determined as the average of about a dozen consistent readings to be 0.5185 when computed for a 0.76 gm. solution. The extinction coefficients for the visible absorption of acid hematin were then all multiplied by such a factor as to bring the average extinction coefficient in the region 5500 to 5580 Ångström units to 0.5185.

The values of the extinction coefficients at the tops of the peaks at 3100 Ångström units were in the case of both curves, oxyhemoglobin and acid hematin, such as to correspond to a 0.76 gm. solution. The ultra-violet portion of these two curves was therefore not corrected further for density. The mid-ultra-violet portion of the oxyhemoglobin curve was corrected for oxygen saturation. The extinction coefficients as determined from the carbon monoxide hemoglobin plates, being determined from solutions of known oxygen capacity, were simply reduced by 8.5 per cent so as to correspond to a 0.76 gm. solution.

The intervals chosen by the various authors for the determination of the spectrophotometric constants are too wide. New intervals having a width of 40 Ångström units might be chosen so as to be centrally located. On the basis of the data herein contained such a set of constants for oxyhemoglobin would be as follows:

Oxyhemoglobin.

$$\begin{aligned} A_0 &= 1.87 && (5580 \text{ to } 5620 \text{ Ångström units}) \\ A'_0 &= 1.14 && (5390 \text{ " } 5430 \text{ " " "}) \\ A''_0 &= 1.068 && (5740 \text{ " } 5780 \text{ " " "}) \end{aligned}$$

Carbon Monoxide Hemoglobin.

$$\begin{aligned} A_c &= 1.343 && (5540 \text{ to } 5580 \text{ Ångström units}) \\ A'_c &= 1.128 && (5360 \text{ " } 5400 \text{ " " "}) \\ A''_c &= 1.128 && (5680 \text{ " } 5720 \text{ " " "}) \end{aligned}$$

Acid Hematin.

$$A_h = 1.465 \text{ (5500 to 5580 Ångström units)}$$

Tables II, III, and IV give the absorption data for acid hematin, oxyhemoglobin, and carbon monoxide hemoglobin in an esti-

mated density corresponding to 0.76 gm. to the liter of oxyhemoglobin and in a layer 1 cm. thick.

In Fig. 1 which gives the data of Tables II to IV, extinction coefficients are converted into transmissions, the extinction coefficients being the cologarithms to the base 10 of the transmissions.

TABLE II.

Extinction Coefficients for Acid Hematin Corresponding to 0.76 Gm. of Oxyhemoglobin per Liter.

Wave-length.	Extinction coefficient.	Wave-length	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.
Å. u.		Å. u.		Å. u.		Å. u.	
2410	2.06	3340	1.4	4950	0.655	5800	0.349
2440	1.8	3400	1.57	5000	0.625	5840	0.315
2460	1.7	3460	1.74	5040	0.61	5865	0.3
2480	1.65	3500	1.85	5070	0.6	5905	0.28
2540	1.59	3540	1.93	5140	0.59	5960	0.26
2600	1.64	3600	2.0	5200	0.588	6030	0.24
2645	1.7	3680	2.05	5260	0.587	6080	0.23
2670	1.74	3760	2.06	5300	0.583	6140	0.222
2720	1.8	3900	2.0	5320	0.58	6200	0.215
2780	1.81	4000	1.94	5360	0.567	6260	0.2125
2830	1.74	4065	1.9	5400	0.548	6300	0.214
2860	1.64	4100	1.88	5420	0.54	6340	0.22
2870	1.6	4200	1.81	5450	0.53	6400	0.24
2890	1.5	4300	1.73	5480	0.522	6460	0.265
2910	1.4	4390	1.63	5500	0.52	6520	0.29
2930	1.3	4440	1.56	5550	0.5185	6560	0.3
2960	1.19	4500	1.45	5580	0.516	6620	0.305
3000	1.07	4560	1.32	5600	0.5125	6660	0.3
3050	0.98	4600	1.23	5640	0.5025	6700	0.285
3100	0.96	4650	1.125	5660	0.494	6730	0.265
3170	0.99	4710	1.0	5680	0.48	6760	0.24
3210	1.07	4760	0.91	5700	0.459	6800	0.205
3230	1.12	4800	0.84	5720	0.43	6850	0.17
3260	1.19	4860	0.75	5740	0.409	6900	0.14
3300	1.3	4900	0.7	5770	0.375	7000	0.11

TABLE III.

Extinction Coefficients for Oxyhemoglobin, 0.76 Gm. per Liter.

Wave-length.	Extinction coefficient	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.
\AA. u.		\AA. u.		\AA. u.		\AA. u.	
2450	2.06	3780	1.25	4750	0.37	5680	0.465
2460	2.0	3800	1.33	4800	0.335	5690	0.5
2480	1.9	3830	1.5	4840	0.31	5700	0.545
2520	1.81	3860	1.75	4920	0.27	5710	0.6
2590	1.77	3890	2.05	4960	0.255	5720	0.655
2660	1.81	3960	3.0	5070	0.233	5730	0.695
2760	1.87	4000	3.7	5120	0.24	5740	0.708
2830	1.81	4040	4.5	5160	0.26	5760	0.713
2870	1.7	4080	5.4	5200	0.295	5770	0.712
2895	1.6	4140	5.7	5240	0.365	5780	0.705
2910	1.52	4200	5.3	5270	0.44	5790	0.665
2930	1.4	4220	4.6	5280	0.462	5800	0.595
2950	1.3	4240	4.0	5300	0.51	5810	0.515
2970	1.2	4260	3.4	5320	0.553	5820	0.465
2985	1.1	4280	2.9	5330	0.58	5830	0.4
3010	1.0	4300	2.5	5340	0.6	5840	0.36
3050	0.9	4330	2.0	5360	0.638	5860	0.265
3100	0.882	4350	1.75	5380	0.66	5880	0.195
3140	0.898	4370	1.5	5400	0.668	5900	0.14
3200	0.98	4400	1.25	5415	0.67	5950	0.087
3230	1.04	4420	1.1	5430	0.667	5960	0.08
3270	1.12	4435	1.0	5440	0.66	5980	0.07
3330	1.21	4450	0.9	5460	0.61	6055	0.05
3400	1.254	4475	0.8	5470	0.58	6140	0.04
3460	1.262	4510	0.7	5480	0.55	6200	0.0355
3520	1.243	4540	0.63	5500	0.495	6300	0.0325
3580	1.155	4570	0.57	5520	0.45	6400	0.03
3600	1.112	4600	0.52	5550	0.42	6500	0.0295
3680	1.065	4650	0.46	5600	0.406	6600	0.0285
3740	1.125	4680	0.43	5650	0.42	6700	0.0275
3760	1.175	4710	0.4	5670	0.445	6800	0.0268

TABLE IV.

Extinction Coefficients for Carbon Monoxide Hemoglobin, 0.76 Gm. per Liter.

Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.	Wave-length.	Extinction coefficient.
Å. u.		Å. u.		Å. u.		Å. u.	
3000	0.81	4080	4.15	4720	0.347	5660	0.658
3040	0.73	4100	4.9	4760	0.33	5680	0.671
3100	0.7	4120	6.0	4800	0.316	5700	0.675
3160	0.745	4130	6.7	4880	0.299	5720	0.671
3200	0.835	4140	7.4	4980	0.289	5730	0.664
3240	0.96	4150	8.0	5040	0.3	5740	0.645
3280	1.083	4160	8.45	5100	0.34	5760	0.575
3340	1.215	4190	8.7	5150	0.393	5770	0.533
3400	1.3	4220	8.44	5200	0.46	5790	0.445
3460	1.333	4230	8.0	5260	0.55	5820	0.325
3520	1.295	4240	7.3	5300	0.61	5840	0.259
3560	1.215	4250	6.5	5320	0.64	5860	0.213
3590	1.1	4260	5.65	5340	0.66	5900	0.164
3620	0.99	4280	4.25	5360	0.671	5950	0.131
3660	0.89	4300	3.0	5380	0.675	6000	0.111
3700	0.825	4320	1.9	5400	0.673	6040	0.1
3760	0.795	4340	1.4	5420	0.665	6140	0.08
3810	0.82	4360	1.1	5440	0.647	6220	0.07
3840	0.89	4380	0.9	5460	0.6125	6360	0.06
3860	0.985	4400	0.8	5480	0.6	6500	0.054
3880	1.1	4440	0.652	5500	0.581	6600	0.052
3900	1.38	4500	0.535	5550	0.565	6700	0.05
3960	1.9	4560	0.461	5600	0.58	6800	0.049
4000	2.4	4600	0.422	5620	0.6	6900	0.048
4040	3.05	4660	0.379	5640	0.63	7000	0.048

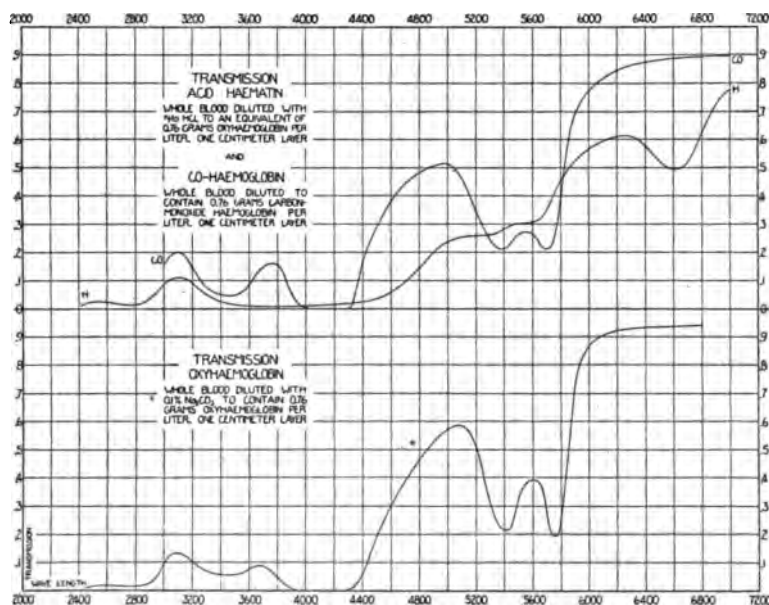


FIG. 1.

DISCUSSION.

Acid hematin is known to have different spectral characteristics under varying conditions as to acid and solvent (4, 5). The curve for material prepared in the manner described is constant and stable, for several days at least. The curve agrees essentially with the spectrogram by Rost, Franz, and Heise (4) of acid hematin prepared in a similar manner and with 1 per cent HCl. The prominent band in the red has its deepest point at about 6620 Ångström units. The difficulty experienced by many (4) in distinguishing photographically the other two bands in the visible is readily understood. They are very shallow. There is a broad general depression extending from 5100 to 5900 Ångström units which is divided by a very slight rise at 5480 Ångström units. In the curve this shows up very distinctly but in a single spectrogram it is discernible only with difficulty, if at all. It first becomes apparent when quantitative absorption measurements are

made. This rise appeared both by the photographic method and with the visual spectrophotometer at Corning.

The great ultra-violet band of acid hematin is broader and shallower than in the case of oxyhemoglobin, the broadening extending it further into the ultra-violet and obliterating the small peak at 3680 Ångström units which appears in the oxyhemoglobin curve. There is beyond the peak at 3100 Ångström units a shallow but distinct band with its center at 2780 Ångström units. Beyond 2400 Ångström units the absorption becomes very great, more rapidly so than in the case of oxyhemoglobin. The further side of this band does not appear within the range of the instrument (2050 Ångström units).

The β -band of oxyhemoglobin is seen to be fainter than the α -band, the difference in depth being, however, slight. As viewed in the ordinary spectroscope there is a subjective difference greater than the real difference. The great ultra-violet band is much deeper, the ratio of the extinction coefficients being about eight. This means that the ultra-violet band can be recognized in eight times as dilute a solution as can the visible bands. A quartz spectrograph is almost necessary.

Oxyhemoglobin has two further bands in the ultra-violet, at 3460 and at 2760 Ångström units separated by a peak at 3100 Ångström units somewhat higher than the corresponding one of acid hematin. The similarity in the ultra-violet of the absorption of the two substances would seem to indicate that it is due in large part to a radical common to both. In fact, this similarity persists, to a less extent, in the visible.

Carbon monoxide hemoglobin has an absorption similar to that of oxyhemoglobin. The differences are interesting; in the visible there is a reduction in the amplitude of the curve, the bands are not so sharp, and the transmission is not so great, particularly at the peak between the α - and β -bands. It is the lowness of this peak which principally accounts for the difference in shade between solutions of the two compounds. The visible bands of carbon monoxide hemoglobin are approximately of the same depth as the β -band of oxyhemoglobin; they are shifted with respect to those of oxyhemoglobin toward the violet; the great ultra-violet band is much deepened and shifted in the contrary direction. In the ultra-violet, the two peaks are higher than in the

case of oxyhemoglobin. The height of the peak at 3760 Ångström units and the depth of the band at 4190 Ångström units decreases very markedly with loss of carbon monoxide from the solution, a change which also holds for oxyhemoglobin.

According to Lewis (25) beyond 3100 Ångström units serum shows a rapidly increasing amount of absorption which reaches a maximum at about 2800 Ångström units, the next minimum being at 2540 Ångström units. The forms of the acid hematin and oxyhemoglobin curves in this region are probably due to the serum present in an approximate dilution of 1:200. In particular the peak at 2540 Ångström units would seem to be due to this. The amount of the extinction due to the admixed serum is only from one-fourth to one-seventh of the whole.

A 1 cm. layer of undiluted blood serum has an absorption which in the red part of the spectrum amounts to an extinction coefficient of about 0.2. This extinction increases uniformly across the visible spectrum to reach a value of about 0.7 in the blue. From the ultra-violet border out to 3200 Ångström units the extinction coefficient of serum is approximately 1. It varies probably with the food taken. I have observed the extremes of 0.8 and 1.7.

In a dilution of 1:200, therefore, serum has an extinction varying from 0.001 to 0.004 in the visible spectrum, being only 0.0015 at its middle point. These figures added to, or subtracted from the extinction coefficients of the hemoglobins do not change them appreciably. The presence of the plasma can therefore be neglected in discussing the visible absorption of these compounds.

The oxyhemoglobin curve as given is approximately that due to a layer of whole blood 0.05 mm. thick. Such a layer is equivalent to a layer of red blood cells in heterogeneous position nine thick, or in rouleau, fifteen thick.

The blood in the superficial capillaries of the skin would therefore be sufficient in quantity to absorb almost completely all the violet and ultra-violet light which might fall upon it. This absorption is particularly intense in the neighborhood of 4200 Ångström units.

Hemotometry.

The banding in the visible of solutions of these three compounds makes it extremely difficult to secure artificial color matches for them. It would be desirable to find an artificial

material whose absorption curve coincided throughout with that of one of them. Under such circumstances the match would appear perfect in any light and to any eye. As the absorption curve of the material to be used as a match deviates more and more from that of the blood preparation, factors are introduced which make it exceedingly difficult to predict from the curves the character which the match will assume.

That a match then exists at all is due to physiological conditions, to the fact that the sum of two colors gives to the eye an impression of a third, a mixed color.

Thus a piece of glass transmitting only green and orange light would in daylight appear to have the same color as a piece of glass transmitting yellow light. But if pure spectral green light were used as an illuminant, the one glass would appear green, the other would be opaque. The two substances may thus appear to have the same color without their absorption curves being at all alike. If their absorption curves are alike, or nearly so, they will appear to have the same color under widely varying conditions, both as to observer and illumination. It is thus essential in order that a color match be satisfactory under varying conditions that the absorption curves of the two substances composing the match be as near alike as possible.

The similarity must be greatest in the region of maximum luminosity, a region whose middle point in the case of ordinary white light is 5560 Ångström units (26). Toward the two ends of the visible spectrum the luminosity is so small that discrepancies here do not materially affect the impression on the eye.

The Miescher hemoglobinometer uses as a match for oxyhemoglobin a gold ruby glass. This glass has a broad absorption band with its center at 5200 Ångström units, which in general balances the two absorption bands of oxyhemoglobin. The transmission of this glass in thickness corresponding to the oxyhemoglobin solution of 0.76 gm. to the liter is given in the upper part of Fig. 2, Curve A. Its absorption only roughly approximates that of oxyhemoglobin. That a match exists at all is, so to speak, accidental, and then only under fixed conditions of illumination.

The upper portion of Fig. 2 contains two other curves, B and C, obtained by multiplying the transmission curves of the Miescher glass and of oxyhemoglobin respectively by the visibility curve

for the Hefner lamp, whose luminosity values are very similar to that of the candle. These two modified curves might be called the physiological transmissions of the two materials. They represent the actual appearance to the eye of each of the two materials when illuminated by the candle. The transmissions of the glass and of the oxyhemoglobin at each point of the spectrum are decreased at each point to correspond to the actual impression which the transmitted light produces on the eye.

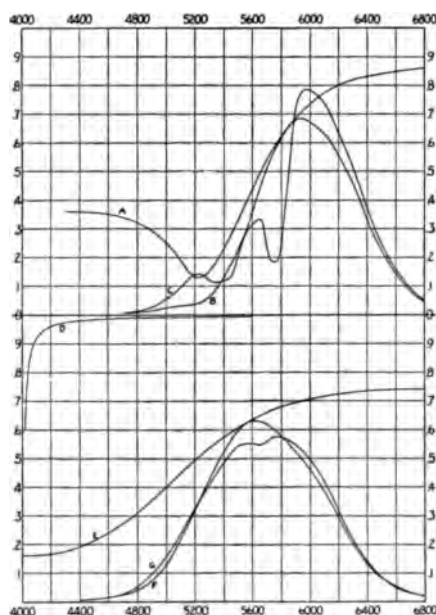


FIG. 2. Curve D is a plot of the equation $xy = -40$, the abscissæ representing time in minutes and the ordinates the percentage short of full color of an acid hematin solution, the squares being each 10 units. For the other curves the ordinates represent transmissions in tenths and the abscissæ wave-lengths in Ångström units. A is the Miescher glass in a thickness corresponding to the oxyhemoglobin of Fig. 1. B is the same multiplied by the visibility of the Hefner lamp. C is the oxyhemoglobin curve multiplied in the same manner. E is the transmission of 1 mm. of high transmission yellow colorimeter glass. F is the same multiplied by the visibility of white light. G is a corresponding acid hematin transmission so multiplied. It is assumed that there is a sufficient intensity of illumination to make applicable the visibility values used.

The amount of agreement or disagreement between these two physiological transmission curves gives an interpretation of the quality of the match. The area under each curve represents the quantity of light which each material appears to transmit. The two areas are equal, the light fields being presumed to balance.

There does not seem to be any way to improve on this match for oxyhemoglobin. There is no red glass that falls to zero transmission near 5800 Ångström units. They all come down to zero much further in the red, presenting a distinctly different hue. It is possible by using an illuminant that is entirely red to confine the problem to the level portion of the high transmission red part of the oxyhemoglobin curve. Then the shades match exactly, but the transmission of the oxyhemoglobin is there so high and the sensitivity of the eye in the middle and far red so low that it is impossible to distinguish any but gross differences in color. The same situation exists in the case of carbon monoxide hemoglobin, except that its relatively low peak between the two visible bands makes it possible to seek a glass whose single band will coincide with the position of this peak. Unfortunately glasses having high transmissions in the blue and red have their central bands too far over in the green. In the case of acid hematin the situation is better.

I have examined at the laboratory of the Corning Glass Works a collection of about 1,000 colored glasses, representing the possibility of a million pairs. There is no glass or combination of glasses giving an absorption whose curve runs closely parallel to that of acid hematin. The "high transmission yellow" semaphore glass made by the Corning Glass Works, however, has an absorption curve which runs smoothly as a mean through the acid hematin curve. It has not been possible to combine this with any other glass so as to produce a combination having a curve more closely approximating that of the acid hematin than does the yellow glass alone.

This glass matches acid hematin best when to its specific absorption is added the flat absorption due to reflection losses at its surface. In colorimetric comparison, therefore, the yellow glass is not to be balanced by a corresponding piece of clear glass, but is to be used alone on one side of the color balance. The hematin solution is, however, to be balanced for reflection

losses at its surface by clear water on the colored glass side of the balance.

This glass in thickness of about 1 mm. is a very close match for acid hemâtin and the discrepancy between its curve and that of the acid hematin is sufficiently small so that the match does not perceptibly vary with individuals. In this thickness the depth of color is about an optimum for judging differences of color depth.

Curve E, Fig. 2, gives the transmission of a piece of this glass 1 mm. thick. Curves F and G are the physiological transmissions of this glass and of a corresponding acid hematin solution respectively. They are obtained by multiplying the transmissions of the two materials by the luminosity curve for white light. It will be seen that they fall more nearly together than do the corresponding Curves B and C for the Miescher hemoglobinometer.

In order that the glass shall match a fixed density acid hematin solution the illumination must be constant. It may be either the light of a northern sky or that given by a nitrogen-filled tungsten lamp filtered through "daylite" Corning glass, the two illuminants being interchangeable. The match is sufficiently good so that further improvement is not a matter of importance, there being a limit to the refinement with which any colorimetric estimation may be made. The use of a fixed thickness of colored material as a standard has a distinct advantage over the use of a wedge. It is impossible to secure an artificial color match which shall run true through a range of thicknesses, though some may do so approximately.

Clinical Estimation of Oxyhemoglobin.

There is no method of estimating hemoglobin which is superior to the oxygen capacity method of Van Slyke (21). For clinical purposes, however, some colorimetric method for the estimation of hemoglobin is essential. It therefore becomes a question of devising a color standard which shall be permanent, which to different individuals shall have the same apparent hemoglobin value, and which can be easily supplied as a uniform product. A stock of "high transmission yellow" glass having the properties set forth in the previous section has been set aside for this pur-

- pose.³ Its hemoglobin value has been carefully estimated. It is to be used in thicknesses of 1 mm. in connection with some colorimeter of the Duboscq type. When used with the Duboscq colorimeter it is placed in the light path of one of the cells (at the top of the plunger) and the corresponding cell or cup is filled with water. In the other cup are placed 5 cc. of 0.1 N HCl (1 per cent c.p. HCl) and to the cup are added 20 c.mm. of blood obtained from a puncture wound with a capillary pipette.³ If the two light fields are now balanced by lowering the plunger in the hematin cup the hemoglobin reading in gm. per 100 cc. of whole blood is represented by the quantity $\frac{0.38d}{t}$ where d is the dilution (in this case 251), t the thickness of the balancing solution in mm., the colorimeter reading, and 0.38 a number empirically determined for a 1 mm. glass.⁴ This expression has to be modified

³ The Arthur H. Thomas Company, Philadelphia, is prepared to supply this glass cut, ground, and polished to thickness, the thickness being engraved on each piece. They will also supply the necessary pipettes, and the "daylite" unit for use at night consisting of an 8" roundel of "daylite" glass and an appropriate shade. The measured glass discs will also be provided as a part of the regular equipment of the new Bausch and Lomb colorimeter of the Duboscq type.

³ The colorimeter described by Bock, J. C., and Benedict, S. R., (*J. Biol. Chem.*, 1918, xxxv, 227) is well adapted for this purpose. The colorimeter cup is small enough to be almost filled by 5 cc. of solution, thus permitting readings to be made on bloods running as low as 20 per cent of the normal without its being necessary to increase the density of the hematin solution by using a larger amount of blood. For the ordinary Duboscq cup the 20 c.mm. of blood suffice for hemoglobin readings down to 50 per cent of the normal.

⁴ Since the thickness of the glass can seldom be exactly 1 mm., there is a further factor multiplying this formula. If h be the thickness of the glass, then for values of h sufficiently near unity it is permissible for this factor to be h itself. As a matter of fact it is slightly less than h . For such an approximation h should not vary from unity by more than 0.05 mm. The actual value of the factor is somewhat involved. If $\phi(\lambda)$, λ being wavelength, represents the values of the extinction coefficients for a 1 mm. glass alone (not including surface loss), then for the glass and surface together $e = \phi(\lambda) + 0.041$ and the transmission is $T = 10^{-\phi(\lambda) - 0.041}$ (Curve E, Fig. 2). The total energy transmitted by the glass in the visible region is the area under this curve, or

$$E = \int_{\lambda=4000}^{\lambda=7000} 10^{-\phi(\lambda) - 0.041} d\lambda$$

because of the fact that the hematin does not reach its full density for some time (as stated above). If x is the time in minutes after making the dilution from the pipette, then the reading is too small by a percentage equal to $\frac{40}{x}$, and the real reading is

$$\frac{38d}{t \left(100 - \frac{40}{x}\right)}$$

It will be seen that when x is very large this becomes equivalent to the first formula given. When x is equal to 10 (at the end of

But, as mentioned above, we are concerned rather with the physiological effect of this energy than with the energy itself. If the visibility of white light is represented by the function $\theta(\lambda)$, then this physiological energy impression or the luminosity of the illuminated glass is

$$P = \int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-\phi(\lambda)-0.041} d\lambda$$

The thickness of the glass being h instead of unity, the new physiological transmission integral is

$$P_h = \int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-h\phi(\lambda)-0.041} d\lambda$$

If $\psi(\lambda)$ represents the extinction coefficient of a solution of acid hematin matching the glass given by $\phi(\lambda)$, then a condition for the match is

$$\int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-\psi(\lambda)} d\lambda = \int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-\phi(\lambda)-0.041} d\lambda$$

and this condition holding, then

$$\int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-h\psi(\lambda)} d\lambda \neq \int_{\lambda=4000}^{\lambda=7000} \theta(\lambda) 10^{-h\phi(\lambda)-0.041} d\lambda$$

but the two integrals are nearly equal when h is nearly unity. No matter how nearly $\phi(\lambda)$ may equal $\psi(\lambda)$ these two latter integrals cannot be alike because of the factor $10^{-0.041}$ in one of them. If conditions are such that the surface factor $10^{-0.041}$ is absent then, since the assumption is that the functions $\phi(\lambda)$ and $\psi(\lambda)$ are not identical, it follows that the integrals also are not identical. They can be made equal for one and only one value of h , a value here chosen as unity. This inequality constitutes sufficient proof of the proposition that a wedge of glass can never exactly balance a varying solution density. The accompanying inequalities in shade constitute the most important difference.

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10 minutes), the hematin has reached to within 4 per cent in 20 minutes to within 2 per cent, and in 40 minutes to within 1 per cent of its final value. For mental calculations the figure given by the simple formula $\frac{0.38d}{t}$ may therefore be simply increased by these percentages.

Table V gives some of the readings by which the calibration of the glass was made and indicates what latitude may be expected in such observations. The table gives results with the artificial illumination and with ordinary daylight. Each column represents ten settings made by the individual whose initials stand at the head of the column. The individuals making the settings were not aware of the readings until they had finished the experiment. It is impossible to expect any single setting to come very close to the mean. This is true of any sort of colorimetric setting though perhaps it is not generally recognized.

A formula giving the oxygen capacity of 100 cc. of blood is obtained by introducing the factor 1.34 into the above formula.

$$\frac{0.38d}{t} \times 1.34 = \frac{0.51d}{t}$$

Haldane and Smith (27) adopt 18.5 cc. oxygen capacity as a normal. The resultant hemoglobin percentage scale is known by his name. On his scale the formula for per cent hemoglobin is $\frac{2.75d}{t}$ per cent.

Tables VI and VII give in convenient form the figures into which t , the thickness of the balancing solution in mm., is to be divided in order to obtain the concentration of hemoglobin in gm. per 100 cc. of whole blood and in per cent Haldane. The dilution is taken as 251. To each of certain possible thicknesses of colored glasses there corresponds a column of figures corrected for the time factor in the formation of acid hematin.

TABLE V.
Colorimeter Readings Balancing a Certain Hematin Solution against Yellow Glass.

[illegible]

Oxygen capacity of blood used.....	=	20.6 cc.
Dilution.....	=	201
Colorimeter factor.....	=	0.369
Thickness of colored glass.....	=	0.97 mm.
Colorimeter factor corrected to 1 mm. glass	=	0.38

TABLE VI.

To obtain gm. of hemoglobin per 100 cc. of whole blood, divide the colorimeter reading into the figure of the appropriate square, the choice of the square depending upon the thickness of the glass used and the length of time since dilution of the blood was made.

Time since dilution.	Thickness of colored glass in mm.										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
min.											
10	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final.....	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

TABLE VII.

To obtain the per cent hemoglobin, Haldane scale, divide the colorimeter reading into the figure of the appropriate square, the choice of the square depending upon the thickness of the glass used and the length of time since dilution of the blood was made.

Time since dilution.	Thickness of colored glass in mm.										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
min.											
10	683	690	697	704	712	719	726	733	740	748	755
15	673	680	687	694	702	709	716	723	730	737	744
20	669	676	683	690	697	704	711	718	725	732	739
30	664	671	678	685	692	699	706	713	720	727	734
40	662	669	676	683	690	697	704	711	718	725	732
Final.....	655	662	669	676	683	690	697	704	711	717	724

CONCLUSION.

The transmissions of acid hematin, oxyhemoglobin, and carbon monoxide hemoglobin for the ultra-violet and visible spectra have been determined to within limits of error, which I believe do not exceed 4 and 2 per cent respectively. The quantitative data for the oxygen capacity of hemoglobin has been shown to agree essentially with certain spectrophotometric data and the two have been brought into correlation with each other and with the complete absorption curves of the three compounds. The optical

conditions underlying colorimetric hemotometry have been briefly discussed. A hemoglobinometer with a colored glass standard has been described.

I am indebted to many for the interest which they have shown in the experimental difficulties which have presented themselves. More particularly I wish to thank Drs. Littleton and Gage of Corning and Dr. Karrer of the United Gas Improvement Company for the facilities which they have extended to me.

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STUDIES IN NUTRITION.

I. THE NUTRITIVE VALUE OF COCONUT GLOBULIN AND COCONUT PRESS CAKE.

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The use of coconut products has increased rapidly in the United States during the last 3 years. Statistics obtained from the Department of Commerce show that during the three 12 month periods ending June 30, in the years 1916, 1917, and 1918, the imports of coconut products were valued at approximately \$8,000,000, \$25,000,000, and \$63,000,000. During the 12 month period ending June 30, 1918, 486,996,112 pounds of copra or dried coconut meats, valued at \$26,945,569, were imported. This is more than four times the quantity imported in 1916.

Copra is pressed in the oil mills of this country in order to obtain coconut oil, which is used to a great extent in the manufacture of vegetable oleomargarine, frequently called nut margarine. After pressing, a cake containing about 10 per cent of oil and 17 to 18 per cent of protein ($N \times 5.7$) remains. In calculating the percentage of protein from the nitrogen content of coconut press cake, the factor 5.7 gives more accurately the percentage of protein than does the factor 6.25, since coconut globulin, which is the chief protein in coconut press cake, contains approximately 17.5 per cent of nitrogen. This is the average percentage of nitrogen found in ten different preparations of coconut globulin.

Coconut press cake is rapidly gaining favor in the United States as a cattle food. It has been found especially valuable when used as a protein concentrate in feeding milch cows. Experiments made both in the United States and in Europe show that its use tends to produce a milk with a high fat content.

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The basic nitrogen of coconut globulin constitutes more than one-third of the total nitrogen, and the percentages of amino-acids in the globulin are as follows:¹

<i>Amino-Acid.</i>	<i>per cent</i>
Cystine.....	1.44
Arginine.....	15.92
Histidine.....	2.42
Lysine.....	5.80

This globulin, therefore, contains all of the basic amino-acids which are known to be essential for normal growth. It also contains tryptophane. Compared with other vegetable proteins it contains a relatively high percentage of lysine which is present in but small quantities in the proteins of most cereal grains. It could be predicted that the protein of the coconut would therefore be of high quality. Nutrition experiments fully corroborate the chemical analyses. The isolated globulin, as well as the press cake, was fed as the sole source of protein to white rats, and normal growth was obtained, showing that the protein has a high biological value.

In the first experiment the rats were fed on a diet of isolated coconut globulin, together with the other essential ingredients of an adequate diet. The necessary salts and water-soluble vitamins were supplied by protein-free milk. This diet was eaten readily, and the rats showed none of the symptoms that accompany a deficient diet. The rate of growth was above normal and Rat 63 gave birth to eight young. The failure to rear them was probably due to the fact that the percentage of protein-free milk in the diet, while sufficient for normal growth of the mother, did not furnish enough water-soluble vitamins for both the mother and young. The curves in Chart I show the high biological value of coconut globulin.

Another experiment with coconut globulin was made, in which the salts were supplied by an artificial mixture approximating in composition the inorganic constituents of milk. The water-soluble vitamins in the diet was supplied by 2 per cent of dried brewers' yeast. As shown on Chart II, the rats grew at an almost

¹ Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149.

normal rate. The 2 per cent of yeast probably supplied scarcely enough water-soluble vitamine for normal growth. It was considered advisable, however, not to increase the percentage of yeast in the diet, in order to eliminate the possibility of adding

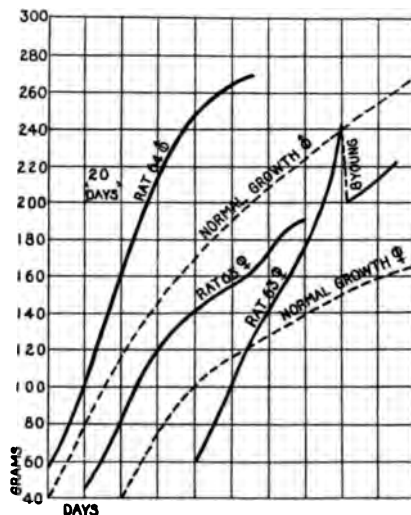


CHART I. Growth on a diet containing coconut globulin as the sole source of protein. More than average normal growth was obtained in each case. Rat 63 ♀ grew more rapidly than the normal rate of growth for a male rat. It was mated with Rat 64 ♂ when about 120 gm. in weight and gave birth to a litter of eight young which were not reared. The composition of the diet was as follows:

	per cent
Coconut globulin*	18
Protein-free milk	28
Agar	5
Starch	21
Butter fat	18
Lard	7

* Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149.

enough yeast protein to exert a supplementing effect on the coconut globulin.

In the third experiment commercial coconut press cake was used as the sole source of protein. The press cake, which was

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ground to a meal, constituted 75 per cent of the diet, the remainder being 21 per cent of butter fat, and 4 per cent of an adequate salt mixture. This diet contained only 13.1 per cent

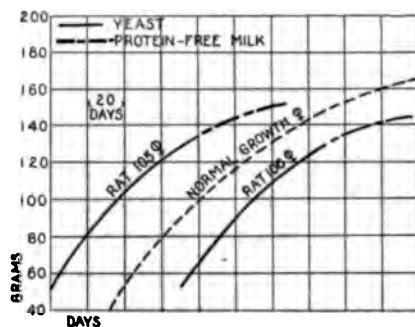


CHART II. Growth on coconut globulin, using 2 per cent of dried brewers' yeast as the source of water-soluble vitamins, and an artificial salt mixture replacing protein-free milk. The rate of growth was below that obtained when protein-free milk was used in the diet (Chart I). The supply of water-soluble vitamins furnished by 2 per cent of yeast was probably scarcely enough for normal growth. The composition of the diet was as follows:

	per cent
Coconut globulin.....	18
Salt mixture*.....	4
Dried brewers' yeast.....	2
Agar.....	5
Starch.....	46
Butter fat.....	18
Lard.....	7

*The composition of salt mixture is as follows (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374):

gm.		gm.
CaCO ₃	134.8	Citric acid + H ₂ O... 111.1
MgCO ₃	24.2	Fe citrate + 1½ H ₂ O.. 6.34
Na ₂ CO ₃	34.2	KI..... 0.020
K ₂ CO ₃	141.3	MnSO ₄ 0.079
H ₃ PO ₄	103.2	NaF..... 0.248
HCl.....	53.4	KAl (SO ₄) ₂ 0.0245
H ₂ SO ₄	9.2	

of protein (N × 5.7). Notwithstanding the relatively low protein content of this diet, several of the rats attained normal growth (Chart III). This experiment shows conclusively the

high biological value of coconut press cake. Because of its bulky character, the diet was far from ideal for nutrition experiments with rats, and would probably be more satisfactory for animals like cattle which have a large digestive tract and are accustomed to coarse, bulky diets. The growth obtained on this diet shows

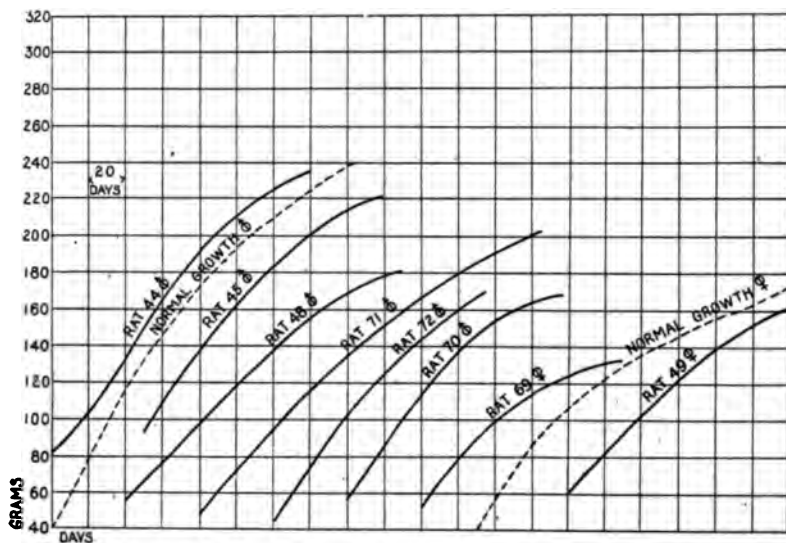


CHART III. Growth on a diet in which coconut press cake was the sole source of protein. The protein content of the press cake was 17.5 ($N \times 5.7$). The diet therefore contained only 13.1 per cent of protein when all the nitrogen in the press cake was calculated as protein nitrogen. Nevertheless the rats grew at an almost normal rate. The composition of the diet was as follows:

	per cent
Coconut press cake.....	75
Salt mixture.....	4
Butter fat.....	21

that coconut press cake contains a sufficient quantity of water-soluble vitamines. There are also indications that it contains some, although an insufficient quantity, of fat-soluble vitamines, since considerable growth was obtained without the addition of butter fat. When the latter was added, the rate of growth became more rapid, as shown in Chart IV.

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Preliminary experiments show that coconut press cake is deficient in inorganic constituents, probably calcium, phosphorus, and chlorine.

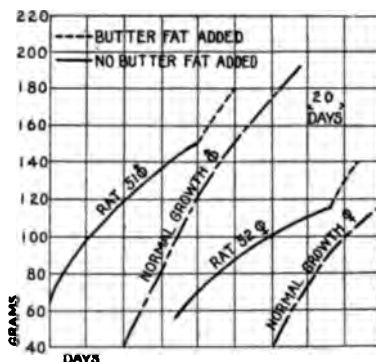


CHART IV. Growth on coconut press cake without the addition of butter fat for a period of 80 days. The growth obtained indicates the presence of some fat-soluble vitamins in coconut press cake. The rate of growth was increased by the addition of butter fat. The composition of the diet during the period when it contained no butter fat was as follows:

	per cent
Coconut press cake.....	75
Salt mixture.....	4
Lard.....	21

During the second period the lard was replaced by butter fat.

CONCLUSIONS.

1. It has been shown that the globulin of the coconut produces normal growth when used as the sole source of protein in an otherwise complete diet.

2. Commercial coconut press cake furnishes the necessary protein for growth at almost a normal rate.

3. Coconut press cake contains sufficient water-soluble vitamins, and experiments indicate that it also contains some fat-soluble vitamins, but the rate of growth is increased by adding butter fat to the diet.

NOTE ON THE DETERMINATION OF BLOOD SUGAR BY THE MODIFIED PICRIC ACID METHOD.

By STANLEY R. BENEDICT.

(From the Department of Chemistry, Cornell University Medical School,
New York City.)

(Received for publication, February 26, 1919.)

In a recent number of this *Journal* Rohde and Sweeney¹ have called attention to the fact that certain samples of the picrate-picric acid solution recommended by the present writer for use in the determination of sugar in blood may fail to precipitate properly proteins or chromogenic substances from the blood. In such instances the final results may be many times too high.

We noted this same fact in our laboratory early this fall, but did not connect it, as Rohde and Sweeney have done, with different samples of picric acid. One of our students made up a solution of the picrate-picric acid solution which failed to precipitate blood properly, though other solutions prepared from the same sample of picric acid worked satisfactorily. A study of the question showed that the important point from the practical standpoint is simply one of the final acidity of the solution. For proper precipitation of the blood the solution must have an acidity as high as 0.05 N or 0.04 N, as determined by titration of a portion of the solution with alkali, using phenolphthalein as indicator. The unsatisfactory solutions are usually alkaline to litmus, due either to impurity in the original sample of picric acid, or to addition of a little too much alkali during preparation of the solution. On account of the difficulty of weighing sodium hydroxide with exactness it is preferable to prepare the 500 cc. of 1 per cent solution by dilution of a 10 or 20 per cent solution.

Titration of the final solution is necessary only when a portion fails to precipitate the blood properly. Such a solution may be corrected by addition of a quantity of glacial acetic acid calcu-

¹ Rohde, A., and Sweeney, M., *J. Biol. Chem.*, 1918, xxxvi, 475.

lated to bring the acidity between 0.05 N and 0.04 N. Any excess of acid over that necessary is to be avoided, since even very small amounts of acetic acid in excess will lead to a remarkable decrease in the rate of ultimate color development, both in blood and in pure glucose solutions.

THE PRESENCE OF CALCIUM IN THE RED BLOOD CORPUSCLES OF OX AND MAN.*

BY DAVID MURRAY COWIE AND HENRIETTA A. CALHOUN.

(From the Department of Pediatrics, University of Michigan, Ann Arbor.)

(Received for publication, September 4, 1918.)

The work of Gryn's, Eykman, Overton, Köppe, Hamburger, and of Hedin,¹ goes to show that the blood cells are impermeable to calcium, strontium, barium, and magnesium.

Marriott and Howland² state that "corpuscles contain no calcium, and that the fibrin clot contains only a very small and fairly constant amount of calcium." On the other hand, the older chemists, Schmidt³ in particular, held that the corpuscles contain appreciable amounts of calcium.

Because of this difference of opinion, we attempted to determine whether the red blood corpuscles contain calcium. Methods have been devised by Laws and Cowie⁴ and by Lyman.⁵ The calcium method for blood using the nephelometer (latest type of Duboscq colorimeter with nephelometer attachment) chosen for the demonstrations was that of Lyman. We make the following slight modifications.

The blood is drawn from the vein in the arm into a 5 cc. pipette attached to a hollow needle by means of a rubber tubing. Before using, the pipette is washed with cold 1 per cent sodium citrate solution made up with 0.9 per cent NaCl solution. This is so successful that, working

* Preliminary report read before the American Pediatric Society, White Sulphur Springs, May, 1917.

¹ Quoted by Hammarsten, O., A text book of physiological chemistry, New York, 5th edition, 1908, 195.

² Marriott, W. McK., and Howland, J., *J. Biol. Chem.*, 1917, xxxii, 233.

³ Schmidt, C., Charakteristik der epidemischen Cholera, Leipsic, 1850; quoted in Mathews, A. P., Physiological Chemistry, New York, 1915, 462.

⁴ Laws, C. H., and Cowie, D. M., *Am. J. Dis. Child.*, 1917, xiii, 236.

⁵ Lyman, H., *J. Biol. Chem.*, 1917, xxix, 169.

rapidly, three 5 cc. pipettes may be filled without removing the needle from the vein.

After the addition of 1 cc. of the 20 per cent sodium acetate solution, the flasks are cooled on ice until a cloud appears. The flasks are corked with well fitting corks that have been soaked in hydrochloric acid solution and washed with distilled water until the wash water gives no test for acid. All the centrifugalizations are at high speed for 3 minutes. The supernatant fluid is poured off the calcium oxalate at the bottom of the tube with a single rapid motion.

Otherwise, the method follows Lyman's directions, and the calculation is made in mg. of *calcium* (not calcium oxide) per 100 cc. of blood. The ammonium stearate solution keeps well. A solution made in June, 1917, is still clear after standing for a year. All chemicals are tested to be sure that they are calcium-free; for the greater part Kahlbaum salts are used.

Ox Blood.—Ox blood is obtained by collecting it directly from the heart into a clean flask surrounded by a freezing mixture of ice and salt. 5 cc. of this are measured as rapidly as possible into Erlenmeyer flasks containing 15 cc. of trichloroacetic acid. The measuring pipette is rinsed in 1 per cent sodium citrate solution to prevent clotting, and the same pipette is used for all the measurements.

Serum and Corpuscles.—To obtain serum and corpuscles from defibrinated blood, fresh blood is defibrinated at once by whipping with a glass stirring rod. Small pieces of clot are subsequently removed by straining through two layers of surgeon's gauze which has been previously rendered calcium-free by soaking in dilute hydrochloric acid solution, and the acid is removed by washing in calcium-free distilled water until no trace of acid is left. This gives a suspension of corpuscles in serum. The corpuscles and serum are separated by centrifugalization. The serum is pipetted off with a suction pipette.

Unwashed Corpuscles.—To the centrifugal tubes from which the serum has been removed 0.9 per cent sodium chloride solution is added. The tubes are washed once to remove serum that may be adherent to the cells, and then centrifugalized for 10 minutes. The supernatant fluid is pipetted off. Comparative analyses are made on the same batch of corpuscles, 5 cc. samples being used.

Washed Corpuscles.—Washed corpuscles are obtained by washing through three changes of 0.9 per cent salt solution. The isotonic solution prevents the removal of adsorbed calcium but favors the removal of absorbed calcium. It requires an infinite number⁶ of washings to remove adsorbed

⁶ Bayliss, W. M., Principles of general physiology, London, 2nd edition, 1918, 69.

substances in isotonic equilibrium. 5 cc. samples of washed corpuscles were used for the calcium determinations.

Following the custom used in obtaining solutions containing a certain percentage of red corpuscles in Wassermann technique, we consider the corpuscles in the bottom of the tube after all the fluid has been pipetted off a constant solution. Any variation between different batches is of minor importance, since the object is not to find a method for determining the calcium content of the blood by using the red corpuscle, but to determine whether calcium is present in the corpuscles.

The serum from undefibrinated blood is obtained by allowing the blood to stand 12 to 24 hours until the serum separates. This serum is decanted and centrifugalized to free it from corpuscles. The serum from the bottom of the vessel may contain enough corpuscles to use for the determination of calcium in corpuscles from undefibrinated blood. If there is any hemolysis, the specimen is discarded.

Our results are given in Table I.

TABLE I.
Calcium Content of Whole Blood in Ox and Man, Mg. per 100 Cc.

Whole blood.	No. of determinations.*	Calcium.
		mg.
Ox blood.		
No. 1.....	11	8.68
" 2.....	10	7.38
" 3.....	7	7.40
Average.....		7.82
Human blood.		
Case 1.....	15	6.64
" 2.....	7	8.92
" 3.....	1	8.12
" 4.....	1	7.82
" 5.....	1	9.08
" 6.....	1	8.82
" 7.....	1	9.06
" 8.....	1	8.79
" 9.....	1	8.82
Average.....		8.45

* For each determination an average of six readings was taken. The unknown was always set at 32.

From Table I it will be seen that three healthy oxen gave an average of 7.82 mg. of calcium per 100 cc. in twenty-eight

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determinations. The mean variation from the average for each animal in no case exceeded 0.5 mg. per 100 cc. Nine healthy men gave an average of 8.45 mg. of calcium per 100 cc. of whole blood in twenty-nine determinations.

The limits of variation of blood calcium in normal individuals are marked, as is shown in the two men, Cases 1 and 2. Lyman⁵ found the same variations in normal individuals, which he was unable to explain. Determinations of the number of corpuscles and of the specific gravity of the blood might aid in clearing up this point by giving an idea of the concentration.

TABLE II.

Calcium Content of Normal Serum and of Serum from Defibrinated Blood in Ox and Man, Mg. per 100 Cc.

Serum.	No. of determinations.	Calcium.
		mg.
Ox 1, undefibrinated*.....	12	8.05
" 1, defibrinated†.....	7	8.42
" 2, undefibrinated.....	5	9.13
" 2, defibrinated.....	3	9.08
Average.....		8.67
Man (Case 2), undefibrinated.....	4	12.07
" (" 2), defibrinated.....	3	12.08
Average.....		12.07

* Blood allowed to clot and serum pipetted off.

† Fibrin removed at once by defibrination.

From Table II it will be seen that two healthy oxen show an average calcium content of 8.67 mg. of calcium per 100 cc. of blood serum in twenty-seven determinations.

One healthy man gave an average calcium content of 12.07 mg. per 100 cc. of blood serum in seven determinations. It is of interest to recall that this man's whole blood calcium content was also high (8.9 mg.).

It will be seen that defibrination of the blood makes no difference in the calcium content of the serum. This also proves that if calcium adheres to the fibrin there is no further increase in the amount adhering after the initial formation of the fibrin threads.

The number of readings on the corpuscles (Table III) would be inadequate if an attempt were being made to determine the amount of calcium actually in the corpuscle itself. But, since the determinations show that the corpuscles constantly contain calcium but in lower concentration than the plasma, we must consider

TABLE III.

Calcium Content of Washed and Unwashed Defibrinated Blood Corpuscles in Ox and Man, Mg. per 100 Cc.

	No. of determinations.	Calcium.
		mg.
Ox 2, unwashed blood corpuscles.....	8	4.98
" 2, washed " "	4	2.07
Man,* unwashed " "	8	4.26
" washed " "	4	3.47

* Case 2.

TABLE IV.

Calcium Partition or Distribution in Ox Blood and in Human Blood.

Portion of blood taken.	Mg. of Ca in 100 cc.	
	Ox.	Man.
Whole blood.....	7.82	8.45
Serum from defibrinated and undefibrinated blood.....	8.67	12.07
Washed corpuscles from defibrinated blood.....	2.07	3.47
Unwashed corpuscles from undefibrinated blood.....	4.98	4.26

that the corpuscle calcium content affects the total amount of calcium in the circulation. To facilitate comparison Table IV is presented.

CONCLUSION.

The red blood corpuscles contain calcium, but in a somewhat smaller concentration than the serum.

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NOTE ON THE STABILIZATION OF DILUTE SODIUM HYPOCHLORITE SOLUTIONS (DAKIN'S SOLUTION).

BY GLENN E. CULLEN AND ROGER S. HUBBARD.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 22, 1919.)

The preparation of dilute sodium hypochlorite solutions electrolytically is so economical and convenient that it was desirable to determine a simple method of stabilizing it for use as Dakin's solution.

We have compared the influence of various substances on the rate of decomposition of dilute sodium hypochlorite solutions.

Dakin^{1,2} set the upper limit of alkalinity of clinical hypochlorite solutions at the end-point of powdered phenolphthalein and used buffer salts to maintain the alkalinity below this limit. He neutralized with boric acid the excess alkalinity of the filtrate from the sodium carbonate and bleaching powder. The original Dakin's solution, therefore, contained both carbonate and borate buffers. Daufresne³ modified Dakin's method by substituting sodium bicarbonate for the boric acid. The resulting solution contained only carbonate buffers.

Cullen and Austin,⁴ studying the alkalinity of dilute sodium hypochlorite solutions containing carbonates, found that the end-point with powdered phenolphthalein was at a hydrogen ion concentration of about 10^{-10} N (pH 10), and that a reduction of the alkalinity below a pH of 9 made the solution too unstable to be of clinical use. The alkalinity of Dakin's solution, therefore, must be between 100 and 1,000 times that of water. The lower limit of alkalinity may be approximately detected by the use of alcoholic solution of phenolphthalein or *o*-cresolphthalein. The desirability

¹ Dakin, H. D., *Brit. Med. J.*, 1915, ii, 318.

² Dakin, H. D., and Dunham, E. K., *Handbook of antiseptics*, New York, 2nd edition, 1918, 116.

³ Daufresne, M., *Presse méd.*, 1916, xxiv, 474.

⁴ Cullen, G. E., and Austin, J. H., *J. Biol. Chem.*, 1918, xxxiv, 553.

of maintaining the alkalinity within this zone has been further emphasized by the observation that dilute hypochlorite solutions with an alkalinity less than indicated by a pH of 9, as well as those with an alkalinity greater than indicated by a pH of 10, are much more irritating than those with a pH between 9 and 10 (Cullen and Taylor).⁵

Daufresne has stabilized electrolytic sodium hypochlorite solutions by the use of small amounts of NaOH.⁶ Although such solutions, unlike those originally described by Dakin, contain no buffer salts, they nevertheless conform to his requirements in concentration and alkalinity, and, therefore, it appears justified to call them Dakin's solutions.

EXPERIMENTAL.

The following substances were tested:

Borates.—Since Dakin used the highly efficient buffer action of borates, it was logical to test the stabilizing influence of borates on electrolytically prepared sodium hypochlorite solutions. Fortunately, the addition of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) so adjusted the reaction that there was no color to powdered phenolphthalein, but definite color with alcoholic phenolphthalein or *o*-cresolphthalein. Borax was added in a concentration of from 0.25 to 1.5 per cent.

Carbonates.—Three sets of carbonate and bicarbonate mixtures, having pH values of 10, 9.5, and 9 respectively, were prepared and added to the test solution in concentrations of 0.5, 1, and 1.5 per cent. McCoy's⁷ formula was used in calculating the ratio of carbonate to bicarbonate.

$$\frac{\text{NaHCO}_3}{\text{Na}_2\text{CO}_3} = C_H \times \frac{0.8}{6.0 \times 10^{-11}}$$

that is, for pH of 10.0 1 molecule Na_2CO_3 to 1.3 molecules NaHCO_3

"	"	9.5	1	"	"	"	4.2	"	"
"	"	9.0	1	"	"	"	13.3	"	"

⁵ Cullen, G. E., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 681.

⁶ Personal communication. Daufresne adds NaOH to the solution in the proportion of 0.2 gm. of NaOH to 5 gm. of NaOCl; i.e., if he prepared a 1 per cent NaOCl solution he would add 0.4 gm. per liter. This solution keeps for months. He uses a special cell with platinum and silver electrodes and probably has much less secondary decomposition produced with this cell.

⁷ McCoy, H. N., *Am. Chem. J.*, 1903, xxix, 437.

Na_2CO_3 alone, of course, makes the solution too alkaline, and NaHCO_3 alone accelerates the rate of decomposition by greatly reducing the alkalinity.

In order to determine the pH of the hypochlorite solutions after the addition of carbonate, 10 cc. samples were decomposed with hydrogen peroxide and titrated with 0.1 N HCl to methyl orange and phenolphthalein. As shown in Table I the pH of the hypochlorite solutions after the addition of 1 per cent carbonate is about 0.2 pH less than calculated for the ratio of sodium carbonate and bicarbonate added.

With impure sodium chloride or with water of high calcium or magnesium content a rather heavy cloud will form in the solution after the addition of carbonate. This will settle out on standing or may be removed by filtration.

TABLE I.

1 Per Cent NaOCl Made from 8 Per Cent NaCl Diluted to 0.5 Per Cent NaOCl with 2 Per Cent Carbonate Solution and Water.

pH of 2 per cent carbonate solution.	Carbonate.	Final pH of hypochlorite solution.
	Final concentration.	
	<i>per cent</i>	
10	0.25	8.9
10	0.5	9.5
10	0.75	9.7
10	1.0	9.8
9.9	0.25	8.7
9.9	0.5	9.2
9.9	0.75	9.6
9.9	1.0	9.7
9.6	0.5	8.8
9.6	1.0	9.4

Sodium Hydroxide.—NaOH was added to the solution in amounts from 0.15 to 0.3 gm. per liter. Over 0.03 per cent makes the solution too alkaline; that is, it gives color with powdered phenolphthalein.

These substances were tested in two ways: First, current was passed through an electrolytic cell, filled with brine, just long enough to produce 0.5 per cent NaOCl. The solution was with-

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drawn from the cell, thoroughly mixed, and measured portions were placed in dark brown bottles containing the desired quantity of hydroxide or buffer. Second, the current was passed until the NaOCl was about 0.7 to 0.8 per cent, that is, near the crest of the curve.⁸ The solution was then removed and diluted to 0.5 per cent with water. In this case the stabilizing substances were added in concentrated solutions. With this procedure more secondary products are formed (see following paper), the solutions are more unstable, and the test, therefore, more severe.

Several experiments were run under varying conditions. In some the solutions were kept in brown bottles; in others they were exposed to light to accelerate decomposition.

Two representative experiments are given in Tables II and III. Any change in the alkalinity of these solutions during the period of the experiments was within the range of alkalinity indicated by the end-points of alcoholic and powdered phenolphthalein and *o*-cresolphthalein.

TABLE II.

10 Liters of 3 Per Cent NaCl. 110 Volts. 14 to 16 Amperes. Initial Temperature 18°C. 15 Minutes. NaOCl=0.5 Per Cent.

Treatment of solution.		Sodium hypochlorite concentration.	
Substance.		Initial.	After 7 days in brown bottle.
	<i>per cent</i>		
Control		0.5	0.35
Borax	0.5	0.5	0.48
"	0.25	0.5	0.465
NaOH	0.3	0.5	0.49
"	0.2	0.5	0.46
"	0.015	0.5	0.42
pH 10 carbonate	1.0	0.5	0.49
" 9.5 "	1.0	0.5	0.45
" 9 "	1.0	0.5	0.41

⁸ Cullen, G. E., and Hubbard, R. S., *J. Biol. Chem.*, 1919, **xxxvii**, 519, Fig. 2, Curve C.

It is evident from our experiments that 0.2 to 0.3 gm. per liter of sodium hydroxide, borax in concentration of 0.25 per cent up, carbonate mixture of pH 10 from 0.5 per cent up, and carbonate mixture of pH 9.5 from 1.0 per cent up, maintain dilute NaOCl solution within the requirements of Dakin's solution for at least a week. The pH 9 carbonate mixture allows somewhat more rapid decomposition. If sodium hydroxide is used, care must be taken that the amount is accurately measured, or the alkalinity may be made dangerously high. The use of borax combines a maximum of convenience and safety.

TABLE III.

10 Liters of 3 Per Cent NaCl. 110 Volts. 20 Minutes. Initial Temperature 7°C. 0.75 Per Cent NaOCl Diluted to 0.50 Per Cent.

Treatment of solution.		Sodium hypochlorite concentration.		
Substance.		Initial.	After 7 days in colorless bottle.	After 26 days in brown bottle.
	<i>per cent</i>			
Control		0.5	0.29	0.09
Borax	1.5	0.5	0.48	0.50
"	1.0	0.5	0.48	0.45
"	0.5	0.5	0.47	0.41
NaOH	0.03	0.5	0.49	0.48
"	0.02	0.5	0.48	
"	0.015	0.5	0.42	0.23
pH 10 carbonate	1.5	0.5		0.49
" 10 "	1.0	0.5	0.49	0.48
" 10 "	0.5	0.5		0.47
" 9.5 "	1.5	0.5		0.48
" 9.5 "	1.0	0.5	0.45	0.47
	0.5	0.5		0.43

DISCUSSION.

The solvent action of the sodium hypochlorite solution is an essential factor in its germicidal efficiency. The necrotic tissue, pus, etc., that protect the bacteria from many antiseptics are dissolved

away by the hypochlorite solution. Fiessinger and his coworkers have attributed this solvent action mainly to the alkalinity of the solution, but Taylor and Austin⁹ have shown that within the zone pH 9 to pH 10 the solvent action is primarily due to the hypochlorite. However, when no alkali was added to the hypochlorite solution and the alkalinity was due entirely to the dissociation of sodium hypochlorite, solvent action ceased when the hypochlorite concentration was reduced below 0.2 per cent.

Solution of the protein material probably takes place in two steps: first, the chlorination of the protein, and second, the formation of soluble sodium salts of the chlorinated proteins. The chlorination of the protein, as indicated by the rate of the decomposition of the hypochlorite solution, is rapid when large amounts of pus and necrotic tissue are present, and the initial 0.5 per cent concentration is maintained in the wound for only a few minutes. When the alkalinity is maintained by buffer salts, the solvent action occurs not only during this period but presumably until all the products capable of reacting are changed to the soluble sodium salts. When the alkalinity is due to small quantities of free alkali, as sodium hydroxide, the concentration of alkali should be sufficient to maintain the alkaline reaction long enough for efficient solvent action. On the other hand, the concentration must not be so great that free alkali remaining unneutralized in the wound is sufficient to cause irritation. The concentration used by Daufresne—0.2 to 0.3 gm. per liter—seems to satisfy these conditions. Experience at the War Demonstration Hospital of The Rockefeller Institute has not yielded clinical evidence to warrant choosing between the two types of hypochlorite solutions provided they conform to Dakin's conditions of hypochlorite concentration and alkalinity.

SUMMARY.

There are two methods of applying Dakin's principle of low alkalinity: first, by maintaining the alkalinity at a pH of 9 to 10 by means of buffer salts, such as carbonate or borates; second, by maintaining a similar alkalinity by means of *small* amounts of alkali. Both methods are efficient.

⁹ Taylor, H. D., and Austin, J. H., *J. Exp. Med.*, 1918, xxvii, 155.

0.5 per cent sodium hypochlorite, prepared by the electrolysis of sodium chloride, may be conveniently stabilized for use as Dakin's solution by the addition of 0.5 per cent borax, of 0.5 to 1.0 per cent of carbonate mixtures of pH 10 to 9.5, or by the addition of 0.2 gm. of sodium hydroxide per liter (Daufresne).



NOTE ON THE ELECTROLYTIC PREPARATION OF DILUTE SODIUM HYPOCHLORITE SOLUTIONS (DAKIN'S SOLUTION).

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(Received for publication, January 22, 1919.)

Although the electrolysis of brine is an old process, information in regard to it is scattered and hidden in the records and patent literature of industrial concerns. Dakin and Carlisle¹ have pointed out its convenience and economy in preparing dilute sodium hypochlorite solutions for disinfection purposes and have designed a simple cell. In instructing army surgeons in the various methods of preparing Dakin's solution, it was desirable for us to determine for ourselves the factors that were of practical importance in its electrolytic preparation. These results are presented for the convenience of other workers.

EXPERIMENTAL.

Choice of Cell.—It seemed desirable to use a cell that could be connected with ordinary 110 volt current, that did not require unusually heavy wiring or power, that was light and strong enough to be transportable with other military hospital equipment, and that was inexpensive. The cell described by Dakin and Carlisle answers these requirements, but we were spared the labor of making this cell by modifying cells already on the market to our purpose. These cells are entirely similar to that described by Dakin and Carlisle, except that the electrode area is smaller in proportion to the volume of solution. The twenty-three intermediate elec-

¹ Dakin, H. D., and Carlisle, H. G., *J. Roy. Army Med. Corps*, 1916, xxvi, 209. See also Dakin, H. D., and Dunham, E. K., *Handbook of antiseptics*, New York, 2nd edition, 1918, 116.

trodes contained 30 square inches of Acheson graphite. The cell² used in these experiments held 10 liters of brine and required between 20 and 35 amperes.

The results are presented as curves, with the omission of the tables from which they were derived.

Influence of Temperature.—In order to determine the effect of the initial temperature of the solution, the current through the cell was maintained constant at 20 amperes by an external resistance.³ This

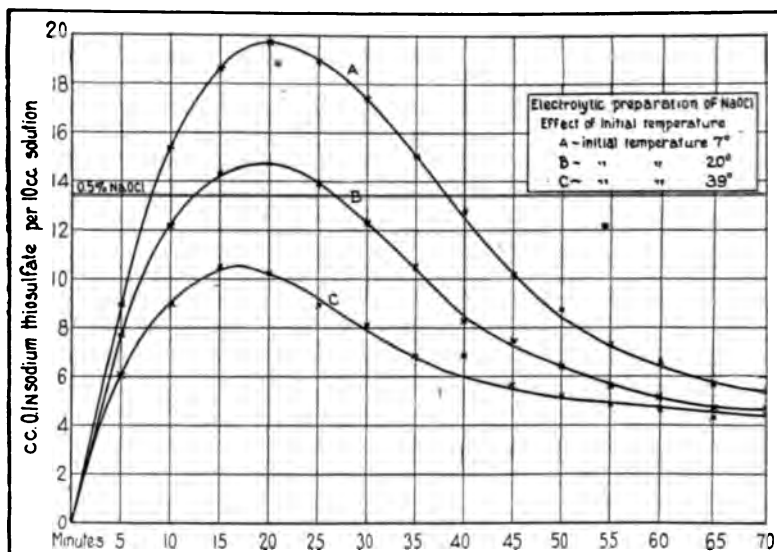


FIG. 1.

eliminates the influence of current fluctuations due to change in internal resistance. Fig. 1 shows the results with initial temperatures of 7, 20, and 39°.

Influence of Salt Concentration.—Increase in salt concentration will, of course, lessen the internal resistance of the cell and consequently increase the production of sodium hypochlorite per unit

²This cell was furnished by courtesy of the Electro Chemical Company of Dayton, Ohio.

³The resistance units used to control the lights in theaters are convenient for this work.

of time. The curves in Fig. 2 show the production of sodium hypochlorite with different salt concentrations under actual operating conditions, with no external resistance.

The 3 per cent solution approximates sea water, and 6 per cent is the strength recommended by the makers. More concentrated solutions may, of course, be used and with them higher concentration of sodium hypochlorite and increased current efficiency may be obtained.

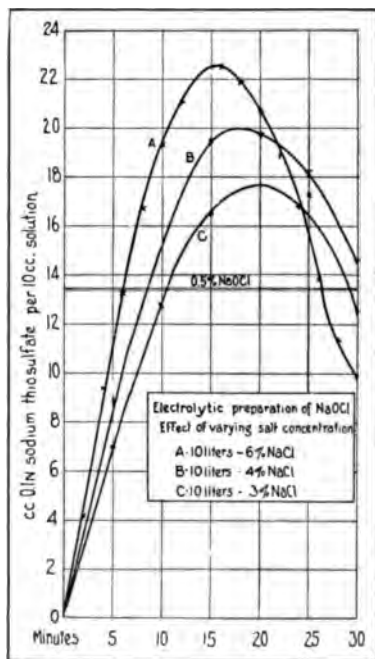


FIG. 2.

Rate of Decomposition of Electrolytic Sodium Hypochlorite.—In the experiments shown above samples of solution were removed from the cell at each of the points shown, titrated, and a portion was set aside for determination of stability. The solution became increasingly unstable with increase in hypochlorite concentration. The results obtained from Curve A, Fig. 1, run at initial temperature of 7°, are plotted on Fig. 3. This increased rate of decomposition is due to increase in secondary products rather than

to the temperature at time of sampling, for in one series in which the samples were all cooled to the same temperature the results were essentially similar.

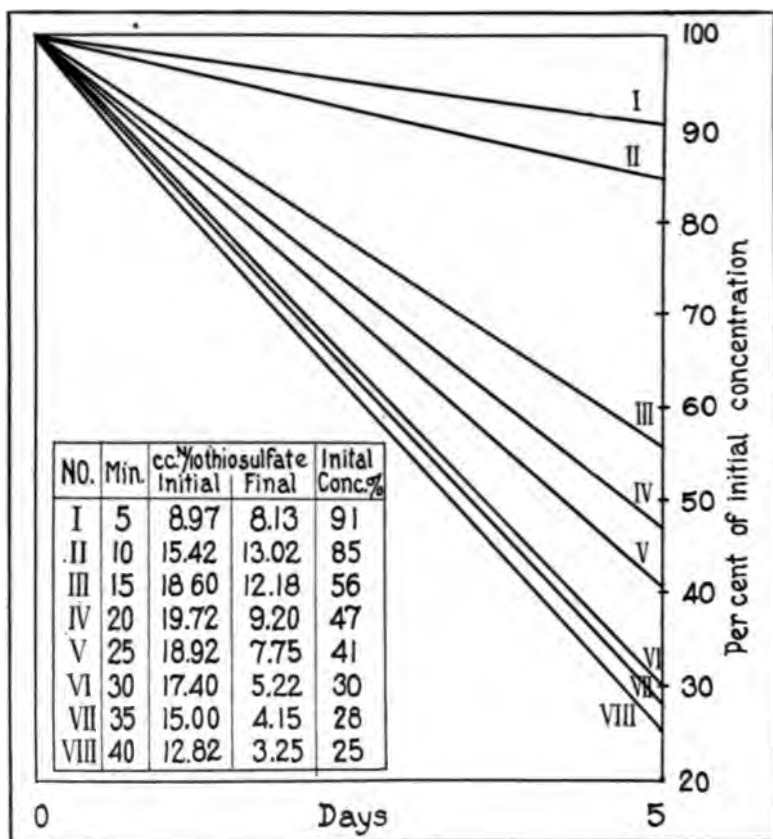


FIG. 3.

DISCUSSION.

It would seem desirable to construct from experimental runs, curves similar to those of Figs. 1 and 2, for each cell. Then, from the initial temperature of the solutions, the time required for a solution of given concentration may easily be determined. Care should be taken that the cell is not operated beyond the peak of the production curve. Ordinarily 3 per cent NaCl (or sea water) is

satisfactory, but if necessary to operate with relatively warm solution the salt concentrations should be increased.

Since the solution, as it comes from the cell, decomposes quickly, it must be stabilized for use as Dakin's solution. As determined in the preceding paper, this can best be accomplished by adding either 0.5 per cent borax, or 0.5 to 1.0 per cent of a mixture of carbonate and bicarbonate of pH 10 to 9.5, or 0.02 per cent sodium hydroxide.

This solution must give no color with powdered phenolphthalein but should give a definite red flash with alcoholic phenolphthalein solution. It should be protected from light and should be titrated frequently.



THE CHEMICAL INVESTIGATION OF SPOILED MEAT.

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The investigations here described are part of a general study of the problems arising in connection with food poisoning. While the cause of such poisonings is not definitely known, it is often associated with bacteria in spoiling food. The symptoms of poisoning are probably due either to toxins produced in the course of the metabolism of the organisms, or to an infection of the intestinal tract. Whatever may be the exact relation of bacteria found in spoiling food to the illness caused by its ingestion, it would be desirable to develop a chemical test by means of which the early stages of spoilage could be detected. This was one of the objects of the present study. Experimentation was limited to spoiled meat, this being the food most frequently suspected of causing poisoning. Also, the experiments were conducted for the most part on samples inoculated with organisms isolated from meat thought to be responsible for actual cases of poisoning. The general method employed was to study the chemical changes taking place in certain components of the meat as a result of the action of the selected organism. The components thus investigated in the meat under observation were: (1) total nitrogen, (2) non-protein nitrogen, (3) ammonia nitrogen, (4) total creatinine nitrogen (creatine plus creatinine), (5) purine nitrogen. Previous investigators studied the changes in meat in cold storage at temperatures below and above freezing.¹ As a rule, the chemical constituents determined differed from those determined in this investigation.

¹ Richardson, W. D., and Scherubel, E. F., *J. Am. Chem. Soc.*, 1908, xxx, 1515; *J. Ind. and Eng. Chem.*, 1909, i, 95. Emmett, A. D., and Grindley, H. S., *ibid.*, 413. Wright, A. M., *J. Soc. Chem. Ind.*, 1912, xxxi, 965. Hoagland, R., McBryde, C. N., and Powick, W. C., *U. S. Dept. Agric., Bull.* 433, 1917.

Analytical Methods.

A rather full description of the methods used in the estimations will be given in the following paragraphs, both on account of the importance of these methods to the experiments here reported and in view of the fact that they may have an interest of their own.

In outline the procedure adopted was as follows: On a small portion of each lot of meat in which spoilage was investigated, the total nitrogen and ammonia nitrogen were first determined. The protein substances were then coagulated in a large sample of the meat and the non-protein substances separated from the coagulum by washing with water. Upon the water solution of the non-protein substances thus separated, determinations were made of nitrogen, purine nitrogen, total creatinine nitrogen, and ammonia nitrogen. It may be noted at this point that the difficulties encountered in applying methods already worked out arose as a consequence of the chemical and physical nature of the decomposed meat. This rendered necessary certain minor changes in the analytical methods originally designed for the fresh product.

Total Nitrogen.—Total nitrogen was determined directly on a 1 gm. sample of the meat by the Kjeldahl-Arnold-Gunning method.

Ammonia. (a) *In meat.*—1 to 3 gm. of uniformly hashed meat were weighed, placed in a large test-tube (8" × 1"), treated with 5 to 15 cc. of water, enough c.p. sodium chloride to form a saturated solution, and 1.2 cc. of 10 per cent sodium hydroxide solution, and aerated for 2 to 3 hours into 10 cc. of 0.1 to 0.01 N acid, the amount of acid depending upon the amount of ammonia present. The excess acid was titrated with standard alkali, using a mixture of methyl red and methylene blue as indicator.²

(b) *In Filtrates containing Non-Protein Nitrogen.*—25 to 40 cc. of the filtrate were treated with sufficient sodium chloride to form a saturated solution, 1.2 to 1.5 cc. of 10 per cent sodium hydroxide solution were added, and the mixture was aerated as in meat.

Separation of Protein from Non-Protein Constituents.—This proved to be the most difficult part of the investigation. As

² The use of this indicator mixture was suggested by Dr. E. M. Frankel who stated that it was devised by Professor Alonzo E. Taylor and Major Caspar W. Miller. It gives an exceedingly sharp color change with 0.01N reagents.

noted above a number of methods previously worked out for fresh meat were tried and while suitable for that, proved unsuccessful when applied to decomposed meat. The method which yielded the most satisfactory results and which was finally adopted consisted in the coagulation of the proteins by means of heat in the presence of alumina cream. The only difficulties encountered in the use of this method were the loss of ammonia during the process of heating and the difficulty of washing the large mass of coagulum and alumina cream free of the non-protein substances. The first of these was overcome by testing a sample of the meat for ammonia before coagulating the protein, while thorough washing of the coagulum according to the technique given below served to avoid the second. The sample of meat (125 gm.) was first finely hashed and added to 400 cc. of boiling water in an aluminium pan with a lip. The meat was broken up by boiling from 1 to 4 minutes, and when fresh meat was used, about 100 cc. of alumina cream were then added. (The alumina cream was prepared by boiling in an open pail a solution of filtered 8 per cent aluminium acetate diluted with about six times its volume of water for 8 to 12 hours, the water being replaced as evaporation occurred. The suspension was then filtered through canvas bags. This method, suggested by Dr. Frankel, is satisfactory and applicable on a large scale.) In badly decomposed meat, 250 cc. of alumina cream were necessary, while meat in intermediate stages of decomposition was treated with an amount of alumina cream between these two limits. After the addition of the alumina cream, the mixture was again brought to the boiling point, stirred, heating continued for another half minute, and the mass then filtered through a large filter paper (15 inches). If the coagulation has been properly carried out, a clear, faintly yellow filtrate is obtained. The mixture was allowed to drain completely and the contents of the filter paper were very carefully returned to the aluminium pan by means of a porcelain spoon. The meat, which had clumped together during the coagulation, was broken apart with the spoon into small bits. About 300 cc. of water were added to the coagulum in the pan, the mixture was brought to a boil, and filtered as before. This washing and scooping out was repeated three times, making four washings in all. Finally, the filter paper and contents were washed once with about 100 cc. of hot water and the filtrate and washings

made up to 3,000 cc. As the meat decomposed, it became more difficult to remove protein hydrolysis products and it was necessary to add a little alumina cream with each boiling. The meat also became very rubbery. If a perfectly clear filtrate was not obtained, the filtrate had to be brought to a boil and more alumina cream used.

In preliminary experiments, the coagulation of the protein was also accomplished by colloidal ferric hydroxide and magnesium sulfate. This method was used upon water extracts of the meat; the procedure was carried out in two ways. In the first, the hydroxide and the magnesium sulfate acted upon an extract from which the particles of meat were not removed. In the second case, the reagents were allowed to act upon the extract after straining out the meat particles. The procedure finally adopted, however, was the one described in detail above, since it was found that by this means the best separation of protein from non-protein material was accomplished.

Creatine plus Creatinine.—Creatine plus creatinine^{3,4} was determined as follows, on a portion of the non-protein nitrogen filtrate equivalent to 1 to 2 gm. of meat: 5 cc. of normal hydrochloric acid solution were added to the portion of the filtrate and the solution was evaporated to about 2 cc. in a 50 cc. conical flask on an electric stove. The flask was then covered with a watch-glass and heated on low heat on the electric plate for 4 to 5 hours to convert the creatine into creatinine at a temperature just below boiling. When cool, the acid was neutralized by a 10 per cent solution of sodium hydroxide, with one drop of a 0.1 per cent methyl red solution as indicator. Immediately upon neutralization, 15 cc. of a pure saturated picric acid solution and 3 cc. of 10 per cent sodium hydroxide solution were added. The solution was then allowed to stand 10 minutes to permit the color to develop, diluted, and compared in a colorimeter with a standard creatinine zinc chloride solution.

Purine Nitrogen.—Purine nitrogen⁵ was determined in the following manner: About two-thirds to three-fourths of the entire

³ Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

⁴ Greenwald, I., and McGuire, G., *J. Biol. Chem.*, 1918, xxxiv, 107.

⁵ Modification of the method of Krüger and Schmid, *Z. physiol. Chem.*, 1905, xlv, 1.

filtrate (usually 2,000 cc.) was first evaporated to 50 cc. under diminished pressure (temperature 40–45°). If necessary, caprylic alcohol was added from time to time to prevent frothing during this evaporation. The concentrated solution was carefully washed into a 250 cc. beaker, the final volume being made up to 100 to 150 cc., and the purines precipitated as the silver magnesium compounds. The precipitation was accomplished by first making the solution distinctly alkaline with ammonium hydroxide. 10 cc. of an ammoniacal silver nitrate solution were then added, this solution being prepared by dissolving 26 gm. of silver nitrate in a liter of water and adding ammonium hydroxide until the precipitated silver hydroxide redissolved. 10 cc. of a 6 per cent disodium phosphate solution were added at this point, and finally 5 cc. of an ammoniacal magnesia mixture solution (100 gm. of magnesium chloride, 200 gm. of ammonium chloride, in 1 liter of water with the addition of ammonium hydroxide until an excess of ammonia was present). Thorough stirring accompanied the addition of each reagent. The solution was allowed to stand 2 hours, filtered on a fluted paper, the precipitate washed four times with a little cold distilled water, and then washed back into the beaker in which the precipitation had been made. The precipitate, consisting of silver-magnesium purine compounds, was then decomposed by adding 10 to 15 cc. of 10 per cent hydrochloric acid solution and allowing to stand in the dark over night. The purine hydrochlorides resulting were filtered into a liter round-bottomed flask, with several bits of cracked porcelain, made slightly alkaline with sodium hydroxide, then distinctly acid with acetic acid and 10 to 15 cc. of 10 per cent acetic acid solution added in excess. The solution was then brought to a boil, and 10 cc. of a 40 per cent sodium bisulfite solution and 10 cc. of a 10 per cent copper sulfate solution successively were added. A white precipitate formed which became brown on boiling. The boiling was continued 3 to 5 minutes. The copper purine compounds were filtered while very hot on a fluted paper and washed thoroughly with hot water. The precipitate was then washed into a Kjeldahl flask and the nitrogen determined by the usual Kjeldahl procedure. It was necessary to add only 15 cc. of sulfuric acid; no potassium sulfate was needed as digestion proceeded rapidly. This method, providing two precipitations, the first in alkaline and the second in

acid solution, gives pure compounds upon which to determine the nitrogen. Experiments were made to determine whether, employing the above procedure, added purines could be recovered quantitatively when allowed to soak up in meat, and it was found that the method gave correct results when purines were thus added.

Total Nitrogen of Filtrate.—This was determined on a 50 cc. portion of the filtrate by the Kjeldahl-Arnold-Gunning method.

Experiments on Meat and Meat Extracts.

The preliminary experiments made on meat, using the methods described, gave the results shown in Table I. In these preliminary experiments, lean meat was hashed and allowed to decompose spontaneously. Each day, after proper mixing, a sample was re-

TABLE I.
Analyses of Fresh and Decomposing Meat.

Date.	Nitrogen percentages of meat.					
	Total N.	NH ₃ -N in meat.	Non-pro- tein N.	NH ₃ -N in filtrate.	Creatine plus creat- inine N.	Purine N.
Series A. Temperature 2-5°C.						
1917						
Dec. 4.....			0.36	0.015	0.12	
" 5.....			0.36	0.017	0.12	
" 6.....			{ 0.30	0.016	0.10	
			{ 0.34	0.015	0.12	
" 8.....			0.35	0.020	0.12	
" 10.....			0.31	0.025	0.12	
" 13.....			0.29	0.055	0.12	
Series B. Temperature 15°C.						
1918						
Feb. 11.....	3.66	0.012	0.43	0.009	0.12	0.027
" 13.....		0.025	0.42	0.030	0.11	0.028
" 15.....		0.043	0.41	0.049	0.13	0.030
" 16.....		0.052	0.40	0.059	0.13	0.028
" 17.....		0.064	0.42	0.070	0.13	0.029
" 18.....		0.077	0.48	0.081	0.14	0.030
" 19.....		0.098	0.44	0.094	0.14	0.029
" 20.....		0.127	0.42	0.11	0.14	0.025
" 21.....	3.80	0.190	0.44	0.13	0.13	0.020

moved from the mass for analysis. The results given in the table are expressed as percentages of the total weight of the meat. Two series of experiments were conducted, Series A at a comparatively low temperature and Series B at a higher temperature.

Owing to the extreme difficulty, if not impossibility, of working with sterile meat, it was decided at this stage of the investigation to employ meat extracts for all further work. This procedure was thought to be justified in view of the fact that the extracts, as prepared in these studies, contained all the substances present in the meat except the insoluble proteins and the lipins. Extracts were accordingly prepared from lean chilled beef, secured 24 to 48 hours after the animal had been slaughtered. The sample of beef was hashed and steeped with an equal volume of water over night. The extract was squeezed through cheese-cloth the following day and, in order to render it approximately sterile, hydrochloric acid was added until the hydrogen ion concentration was between 10^{-3} and 10^{-4} N. The acidified liquid was then allowed to stand for 1 or 2 hours. Bacterial cultures on agar plates showed that such a medium was fairly effective for killing off most of the microorganisms, the counts varying from 5 to 20 per cc. Before inoculating with test organisms, the acid extract was made alkaline with sodium hydroxide. A voluminous precipitate of acid protein was produced by the hydroxide. Addition of alkali was continued until the hydrogen ion concentration was between $10^{-6.5}$ and 10^{-7} N. The extract was then diluted with sterile water to bring the concentration of sodium chloride to about 0.6 to 0.7 per cent.

This extract was transferred to flasks, with the customary aseptic precautions, approximately the same amount being introduced into each flask. The contents of the flasks were inoculated with 10 cc. each of a broth culture having a rich growth of the test organism. At intervals, usually once a day, a complete analysis was made on the contents of each flask by the methods already described. At the end of the experiments, a drop of the extract was in every case found to be teeming with the organism studied. No evidence of contamination was obtained. Presumably, the organism used for inoculation was numerically so much greater than the few in the extract before inoculation that the latter were killed off. For all practical purposes, therefore, the effects noted in these studies were those of a pure culture. In order to follow

the changes produced in the meat extracts, cultures were kept at room temperature (15–25°), although the optimum temperature for the growth of the organisms studied is 38°.

TABLE II.
Action of Bacillus proteus on Meat Extract.
Experiment A.

	Gm. per liter.							
	Mar. 26	Mar. 28	Mar. 29	Apr. 1	Apr. 2	Apr. 3	Apr. 4	Apr. 5
Total N.....		1.06	1.04					
Ammonia N.....	0.024	0.027	0.015	0.030	0.031	0.040	0.046	0.056
Non-protein N.....	0.499	0.487	0.469	0.478	0.444	0.509	0.485	0.490
“ “ minus								
NH ₃ -N.....	0.475	0.460	0.454	0.448	0.413	0.469	0.439	0.434
K ₁ plus K ₂ N.....	0.145	0.139	0.142	0.139	0.136	0.131	0.131	0.124
Purine N.....	0.034	0.038	0.033	0.036	0.036	0.029	0.028	0.022
Residual non-protein N.....	0.296	0.283	0.279	0.273	0.241	0.309	0.280	0.288

TABLE III.
Action of Bacillus proteus on Meat Extract.
Experiment B.

	Gm. per liter.						
	Mar. 26	Mar. 28	Mar. 29	Apr. 1	Apr. 2	Apr. 4	Apr. 5
Total N.....		1.06	1.06				
Ammonia N.....	0.026	0.025	0.017		0.031	0.044	0.051
Non-protein N.....	0.513	0.501	0.481		0.476	0.497	0.488
“ “ minus							
NH ₃ -N.....	0.487	0.476	0.464	0.463	0.445	0.453	0.437
K ₁ plus K ₂ N.....	0.129	0.142	0.139	0.139	0.139	0.131	0.124
Purine N.....	0.035	0.037	0.036	0.035	0.037	0.028	0.024
Residual non-protein N....	0.323	0.297	0.289	0.289	0.269	0.294	0.289

In the first series of experiments with *Bacillus proteus*, a different procedure from the one just described was employed which, however, did not prove entirely satisfactory and was not used in any other experiment. In this series the extraction was made with a 3 per cent salt solution to remove a higher percentage of the globulin in the meat, sterilized by adding about 8 per cent of ether, shaken,

and allowed to stand 24 hours. The ether was then removed by aspiration with sterile air and the extract diluted with enough sterile water to bring the sodium chloride concentration to about 0.9 to 1.0 per cent. The objections to this method of sterilization were

TABLE IV.

Action of Bacillus coli communis on Meat Extract.

	Gm. per liter.									
	Apr. 10	Apr. 11	Apr. 12	Apr. 13	Apr. 15	Apr. 16	Apr. 17	Apr. 18	Apr. 19	
Total N.....	4.82									
Ammonia N.....	0.086	0.096	0.099	0.100	0.193	0.386	0.756	1.16	2.08	
Non-protein N.....	1.43	1.37	1.44	1.68	1.80	2.13		2.77	3.48	
“ “ minus										
NH ₃ -N.....	1.34	1.27	1.34	1.58	1.61	1.75		1.61	1.40	
K ₁ plus K ₂ N.....	0.554	0.555	0.523	0.506	0.588	0.481	0.321	0.303	0.085	
Purine N.....	0.109	0.123	0.122	0.147	0.120	0.054	0.008	0.001	0.0002	
Residual non-protein N.....	0.677	0.592	0.695	0.927	0.902	1.21		1.31	1.31	

TABLE V.

Action of Bacillus paratyphosus β on Meat Extract.

Strain 180.

	Gm. per liter.									
	Apr. 20 7 p.m.	Apr. 22 10 a.m.	Apr. 23 6 p.m.	Apr. 25 10 a.m.	Apr. 26 10 a.m.	Apr. 27 10 a.m.	Apr. 28 10 a.m.	Apr. 29 3 p.m.	May 1 3 p.m.	May 7 3 p.m.
Total N.....	4.75									
Ammonia N.....	0.083	0.090	0.112	0.133	0.176		0.361	0.706	0.482	1.32
Non-protein N...	1.64	1.66	1.17	1.47	1.71		2.29	2.37	2.32	2.88
“ “										
minus NH ₃ -N...	1.56	1.57	1.06	1.34	1.53	1.71	1.93	1.66	1.84	1.56
K ₁ plus K ₂ N.....	0.521	0.514	0.494	0.496	0.540	0.540	0.560	0.554	0.446	0.427
Purine N.....	0.118	0.135	0.121	0.102	0.103	0.111	0.100		0.103	0.049
Residual non-protein N.....	0.921	0.921	0.445	0.742	0.887	1.06	1.27		1.29	1.08

the difficulty of removing the ether and the fact that dilution of the extract to reduce the salt concentration to 0.9 to 1.0 per cent yielded a very dilute broth. Although *Bacillus proteus* is a vigorous organism, this broth did not furnish a suitable substratum for its growth.

Organisms Studied.—*Bacillus proteus*, *Bacillus coli communis*, *Bacillus paratyphosus* β (two strains which had caused meat poi-

TABLE VI.
Action of Bacillus paratyphosus β on Meat Extract.
Strain 222.

	Gm. per liter.									
	Apr. 20 1 p.m.	Apr. 22 10 a.m.	Apr. 23 6 p.m.	Apr. 25 10 a.m.	Apr. 26 10 a.m.	Apr. 27 10 a.m.	Apr. 28 10 a.m.	Apr. 29 3 p.m.	May 1 3 p.m.	May 7 3 p.m.
Total N.....	4.81									
Ammonia N.....	0.083	0.094	0.116	0.142	0.192		0.401	0.434	0.482	1.64
Non-protein N....	1.64	1.42	1.31	1.50	1.78		2.43	2.50	2.31	3.13
“ “										
minus $\text{NH}_3\text{-N}$...	1.56	1.33	1.19	1.45	1.59	1.73	2.03	1.87	1.83	1.49
K_1 plus K_2N	0.520	0.540	0.513	0.520	0.534	0.540	0.554	0.560	0.560	0.514
Purine N.....	0.118	0.132	0.123	0.093	0.098	0.097	0.104	0.099	0.102	0.080
Residual non- protein N.....	0.922	0.658	0.554	0.837	0.958	1.09	1.37	1.21	1.17	0.894

TABLE VII.
Action of Bacillus enteritidis (Gaertner Bacillus) on Meat Extract.
Strain 25.

	Gm. per liter.									
	Series 1.					Series 2.				
	June 12	June 15	June 16	June 17	June 18	June 29	June 30	July 1	July 2	July 3
Total N.....	6.30				6.30					
Ammonia N.....		0.059	0.590	0.829	1.50		0.070	0.138	0.351	0.533
Non-protein N....		1.86	3.92	4.69	6.08		0.908	1.02	1.26	1.36
“ “										
minus $\text{NH}_3\text{-N}$...	1.76	1.80	3.33	3.86	4.58	0.855	0.838	0.881	0.907	0.829
K_1 plus K_2N	0.704	0.670	0.192	0.384	0.253	0.308	0.312	0.261	0.174	0.134
Purine N.....	0.133	0.155	0.115	0.110	0.045	0.057	0.063	0.058	0.047	0.041
Residual non- protein N.....	0.923	0.975	3.02	3.37	4.28	0.490	0.463	0.562	0.686	0.654

soning), *Bacillus enteritidis* (Gaertner bacillus, two strains), *Bacillus subtilis*, and *Streptococcus brevis* were the types used in the experiments. The sources of these organisms will be given elsewhere.*

* Greenwald, I., unpublished data.

TABLE VIII.
Action of *Bacillus enteritidis* (Gaertner *Bacillus*) on Meat Extract.
Strain 132.

	Gm. per liter.									
	Series 1.					Series 2.				
	June 14	June 15	June 16	June 17	June 18	June 29	June 30	July 1	July 2	July 3
Total N.....	6.15				6.15					
Ammonia N.....		0.046	0.604	0.819	1.50		0.058	0.116	0.292	0.481
Non-protein N....		1.88	3.57	4.59	6.20		0.940	1.03		1.36
“ “										
minus NH ₃ -N...	1.76	1.83	2.97	3.77	4.70	0.855	0.882	0.917		0.874
K ₁ plus K ₂ N.....	0.704	0.673	0.105	0.300	0.205	0.308	0.306	0.253	0.174	0.162
Purine N.....	0.133	0.141	0.035	0.057	0.005	0.057	0.061	0.066	0.056	0.030
Residual non-protein N.....	0.923	1.02	2.83	3.41	4.49	0.490	0.515	0.598		0.682

TABLE IX.
Action of *Bacillus subtilis* on Meat Extract.

	Gm. per liter						
	July 15	July 16	July 17	July 18	July 19	July 20	July 21
Total N.....	4.58						
Ammonia N.....	0.054	0.096	0.738	1.14	1.36	1.75	2.02
Non-protein N.....	1.16	1.33	2.49	2.90	2.97	3.33	3.68
“ “ minus							
NH ₃ -N.....	1.11	1.23	1.75	1.76	1.61	1.58	1.66
K ₁ plus K ₂ N.....	0.394	0.151	0.408	0.196	0.164	0.078	0.048
Purine N.....	0.064	0.064	0.074	0.050	0.040	0.016	0.033
Residual non-protein N.....	0.652	1.02	1.27	1.51	1.41	1.49	1.58

TABLE X.
Action of *Streptococcus brevis* on Meat Extract.

	Gm. per liter.						
	July 15	July 17	July 18	July 19	July 20	July 21	July 22
Total N.....	4.56						4.56
Ammonia N.....	0.054	0.32	0.90	1.29	1.59	1.75	2.16
Non-protein N.....	1.16	1.98	2.60	2.92	3.40	3.42	3.80
“ “ minus							
NH ₃ -N.....	1.11	1.66	1.70	1.63	1.81	1.67	1.64
K ₁ plus K ₂ N.....	0.394	0.368	0.225	0.218	0.130	0.122	0.152
Purine N.....	0.064	0.072	0.068	0.060	0.004	0.035	0.042
Residual non-protein N.....	0.652	1.22	1.40	1.35	1.68	1.51	1.46

Most of the organisms were the colon-typhoid group, which are the organisms chiefly concerned in food poisoning. For comparison, it was desired to determine whether organisms belonging to a totally different group produced changes in the substratum similar to those produced by the colon-typhoid group. For this purpose *Streptococcus brevis* and the spore-forming *Bacillus subtilis* were used. It should be noted in the case of *Bacillus enteritidis*, that the organism grew very rapidly so that the early stages of decomposition were not obtained. The experiments were, therefore, repeated to determine the intermediate changes. These results are given in Table VII under Series 1 and 2 respectively.

The results of the experiments on meat extracts are presented in Tables II to X. The nitrogenous constituents are the most important for determining the nature of the changes occurring in the spoiling meat; Figs. 1 to 9 show the nitrogen content of the various nitrogen-bearing fractions of the extract as percentages of the total nitrogen. The times of action plotted along the abscissæ are not plotted according to scale in some of the figures. For the purposes in view, the results are plotted in equal increments although some of the time intervals were different.

DISCUSSION.

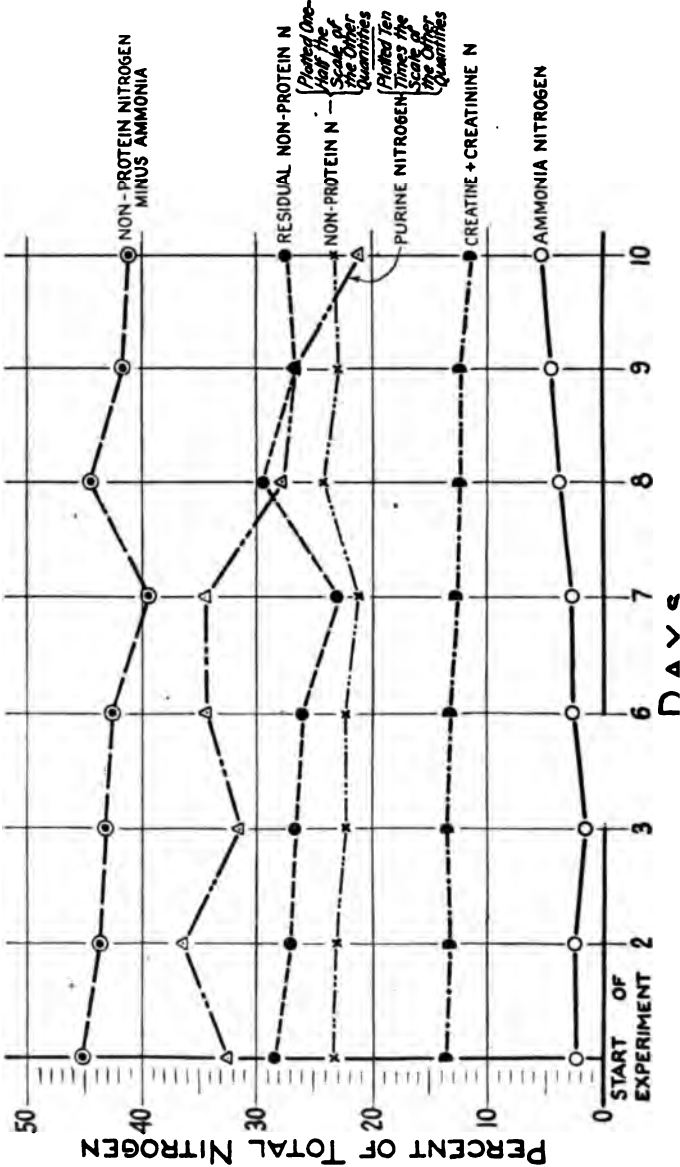
The results presented in the tables and the figures show some marked differences as well as similarities by the actions of the different strains. The creatine-creatinine values change only slightly with *Bacillus proteus* and *Bacillus paratyphosus* β , but decrease markedly with *Bacillus coli communis*, *Bacillus enteritidis*, *Bacillus subtilis*, and *Streptococcus brevis*. The purine values decrease rapidly with *Bacillus coli communis* and *Bacillus enteritidis*. With *Bacillus proteus* and *Bacillus paratyphosus* β the purine nitrogen showed a more or less irregular (accidental) variation for the first action followed by a small decrease. *Bacillus subtilis* and *Streptococcus brevis* gave a distinct final increase in the purine indicating a synthesis. The ammonia increased in every case. The non-protein nitrogen and residual non-protein nitrogen values represent the summation of the changes of a number of substances.

The results show that bacteria exert certain selective actions on definite substances or groups of substances. The results are

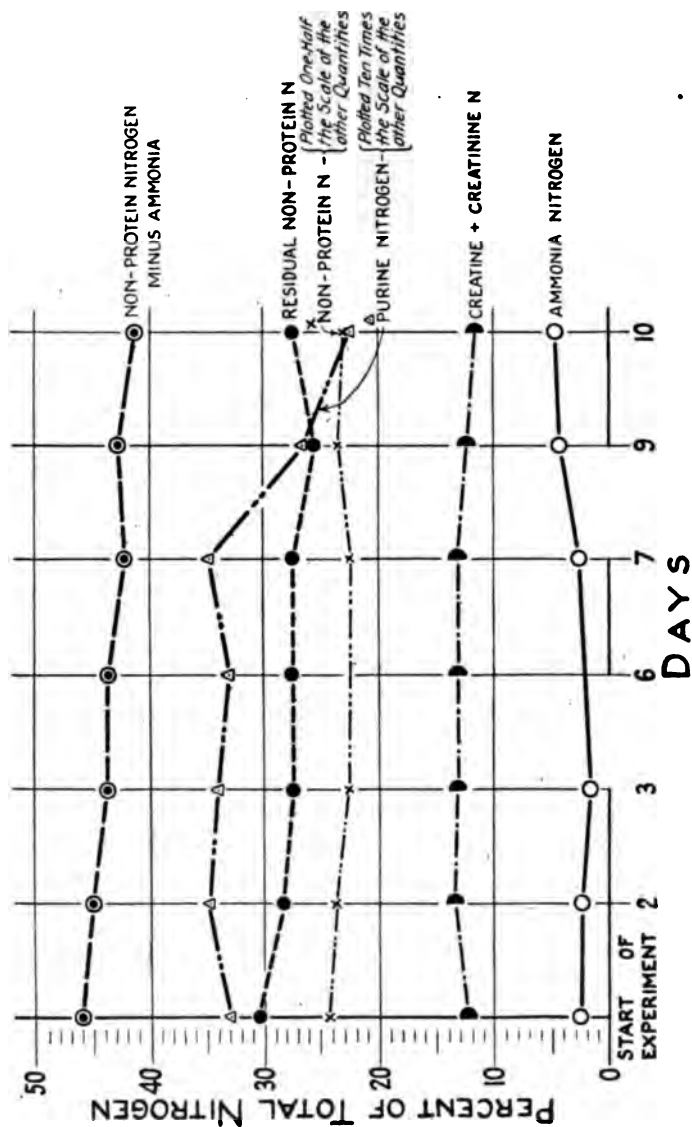
not as yet quantitative enough for careful comparisons, but indicate that more careful determinations in which the conditions are controlled more accurately and with synthetic media, wholly or in part, would give a method for distinguishing various strains of bacteria.

The increase in the ammonia content, which is the one common factor in all the actions, may be of value in determining when the meat becomes unpalatable or unsuitable for use by estimating the amount of this constituent present. This question is taken up in the paper which follows.

ACTION OF B. PROTEUS ON MEAT EXTRACT-EXPERIMENT A
(EXPRESSED AS PERCENTAGES OF TOTAL NITROGEN)



ACTION OF *B. PROTEUS* ON MEAT EXTRACT-EXPERIMENT B (EXPRESSED AS PERCENTAGES OF TOTAL NITROGEN)



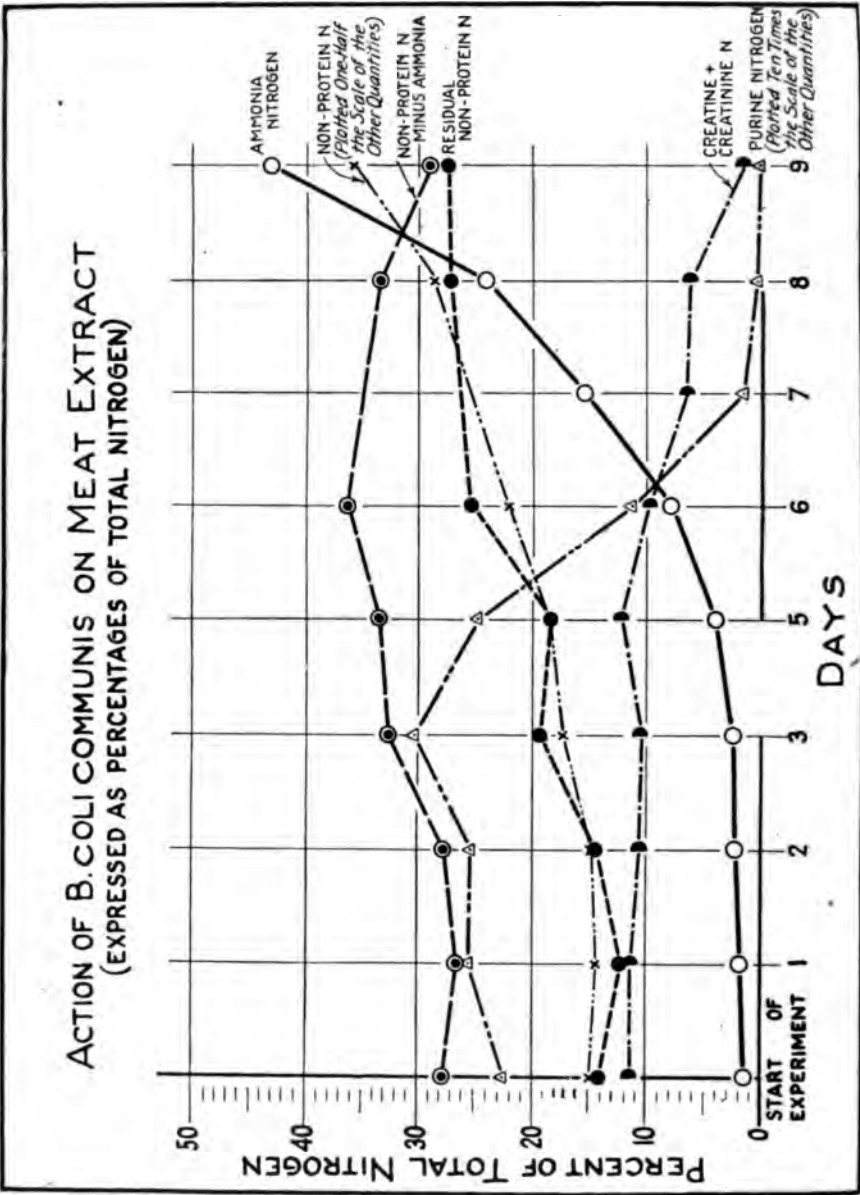


FIG. 8.

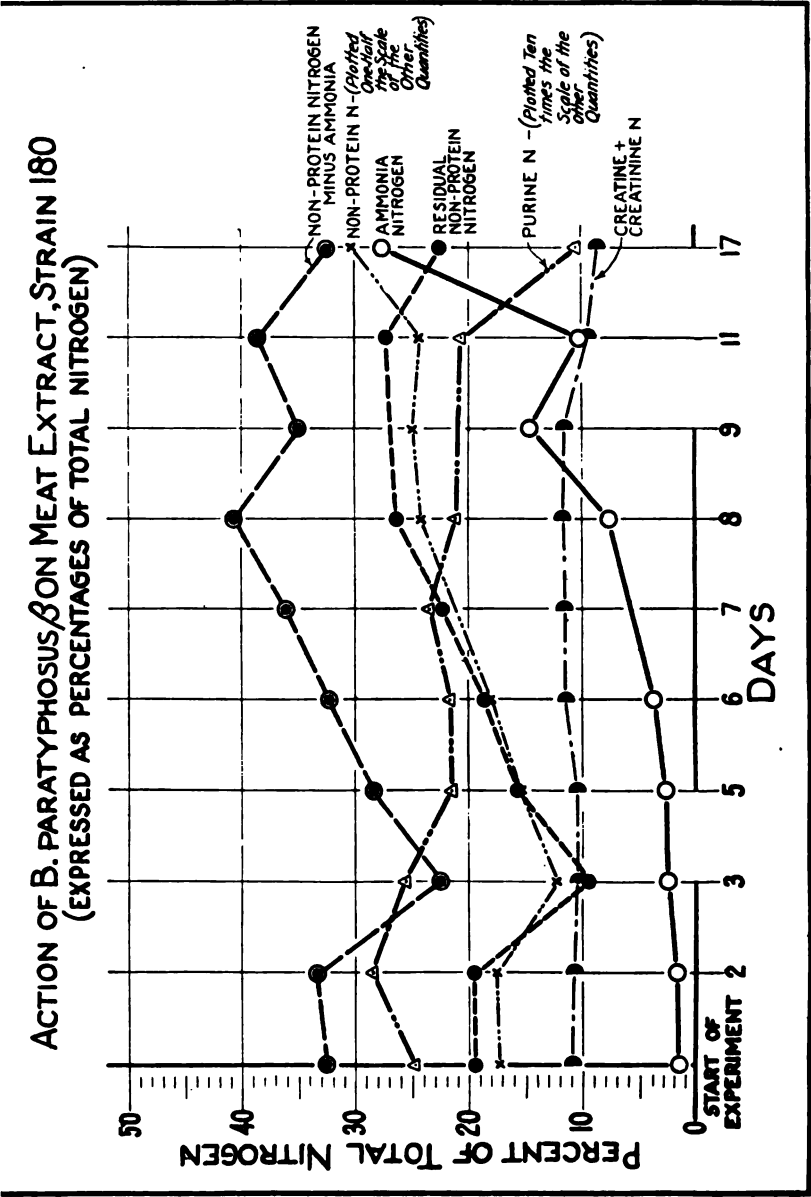
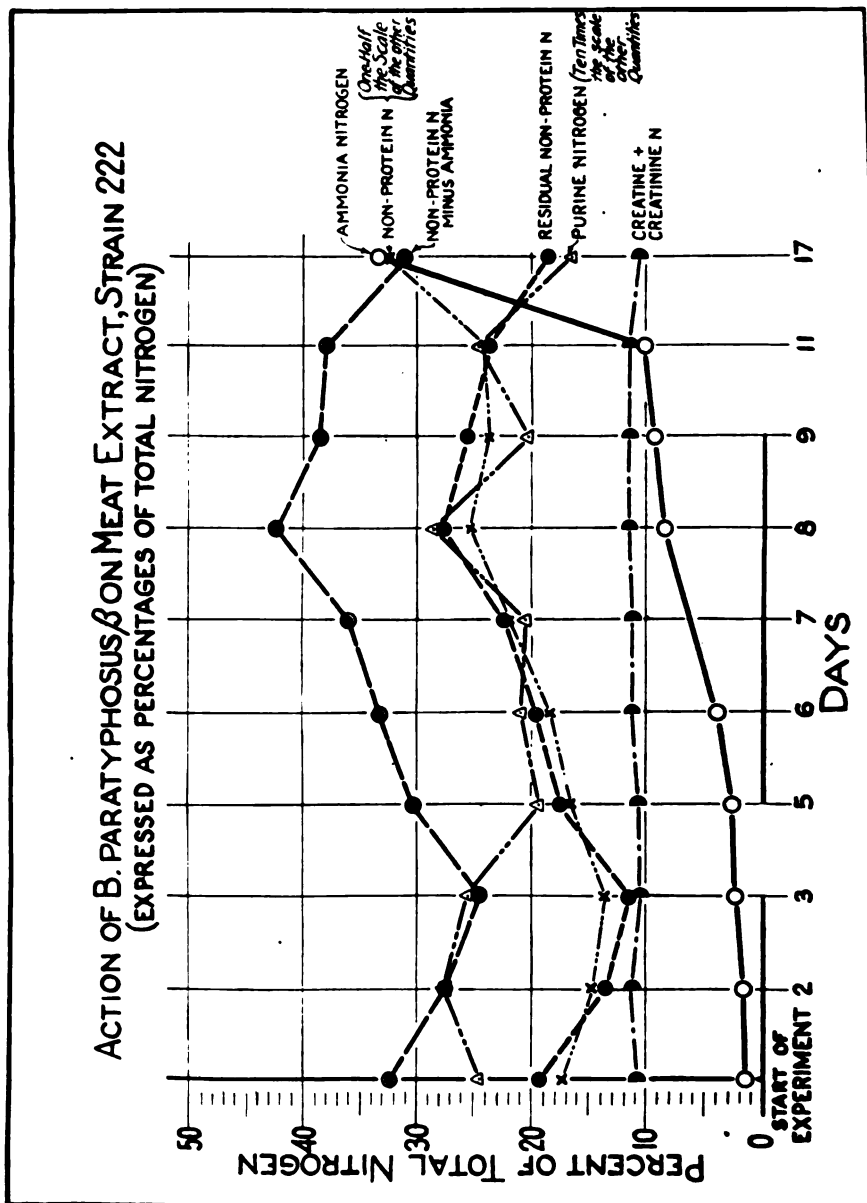


FIG. 4.



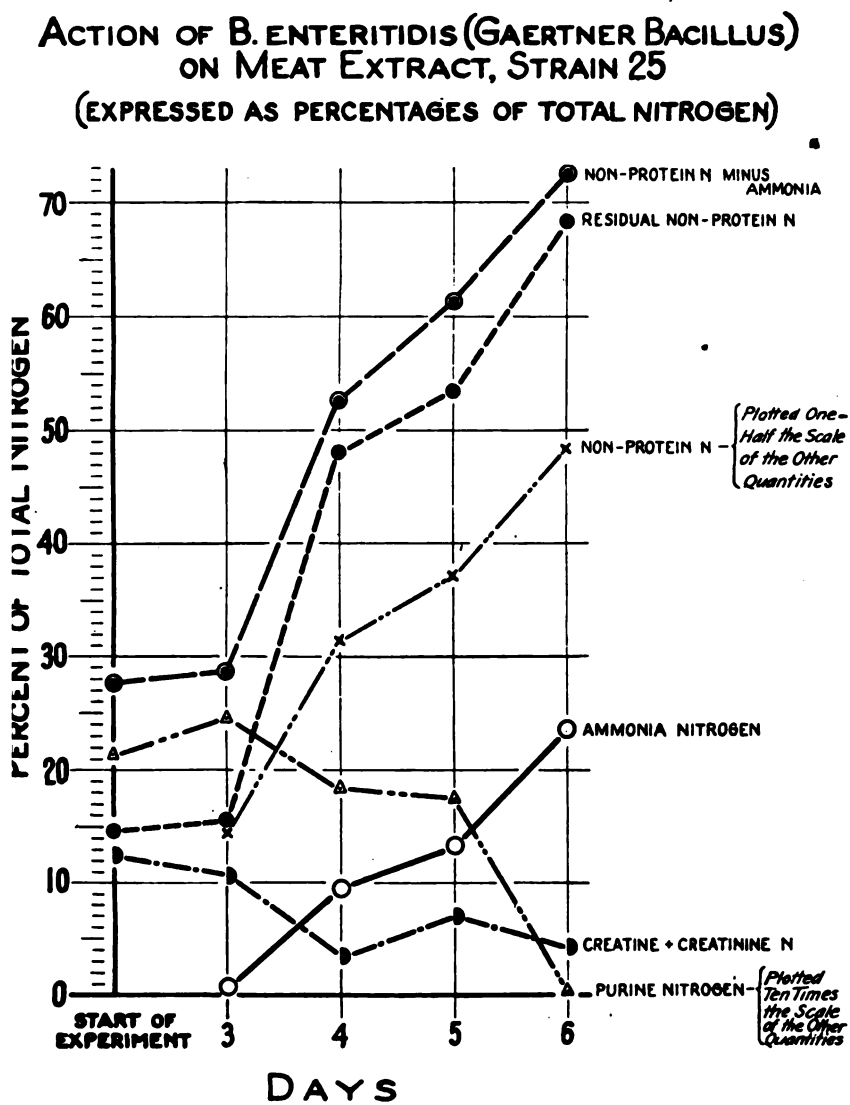


FIG. 6.

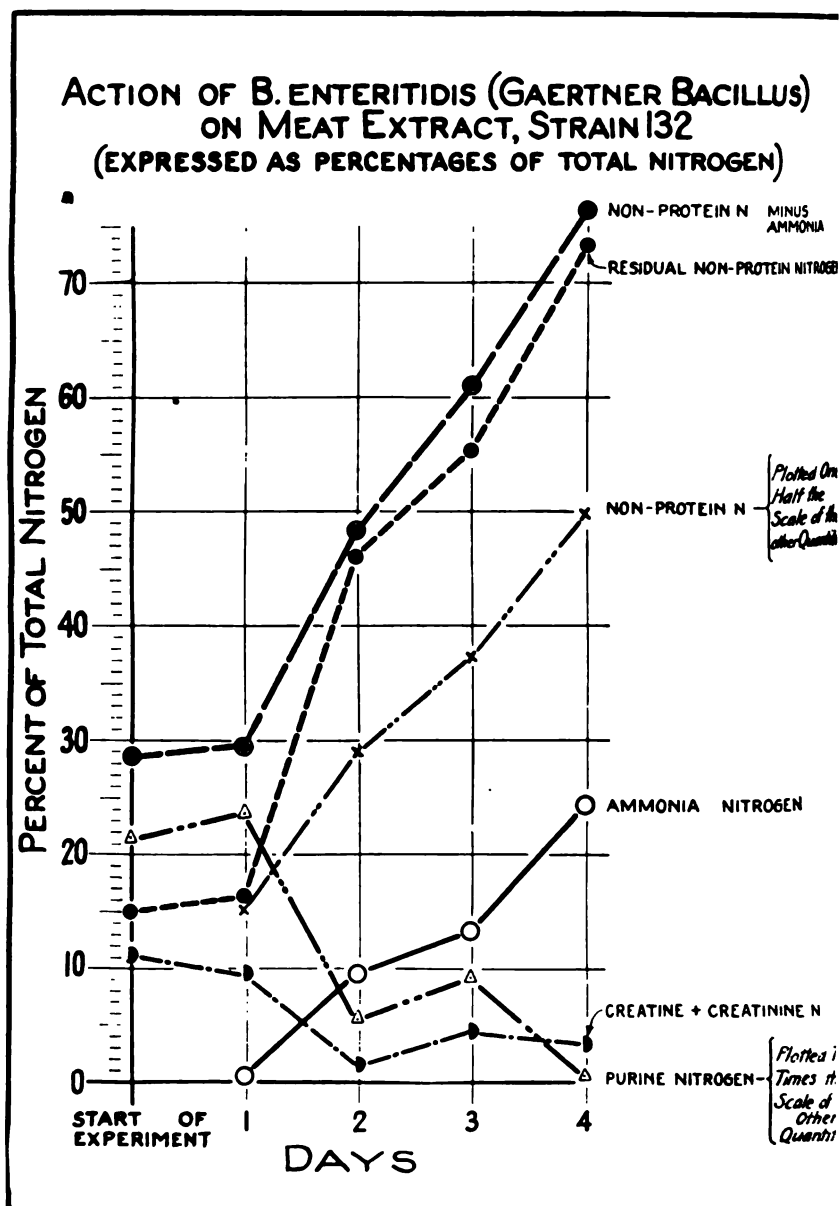


FIG. 7.

**ACTION OF *B. SUBTILIS* ON MEAT EXTRACT
(EXPRESSED AS PERCENTAGES OF TOTAL NITROGEN)**

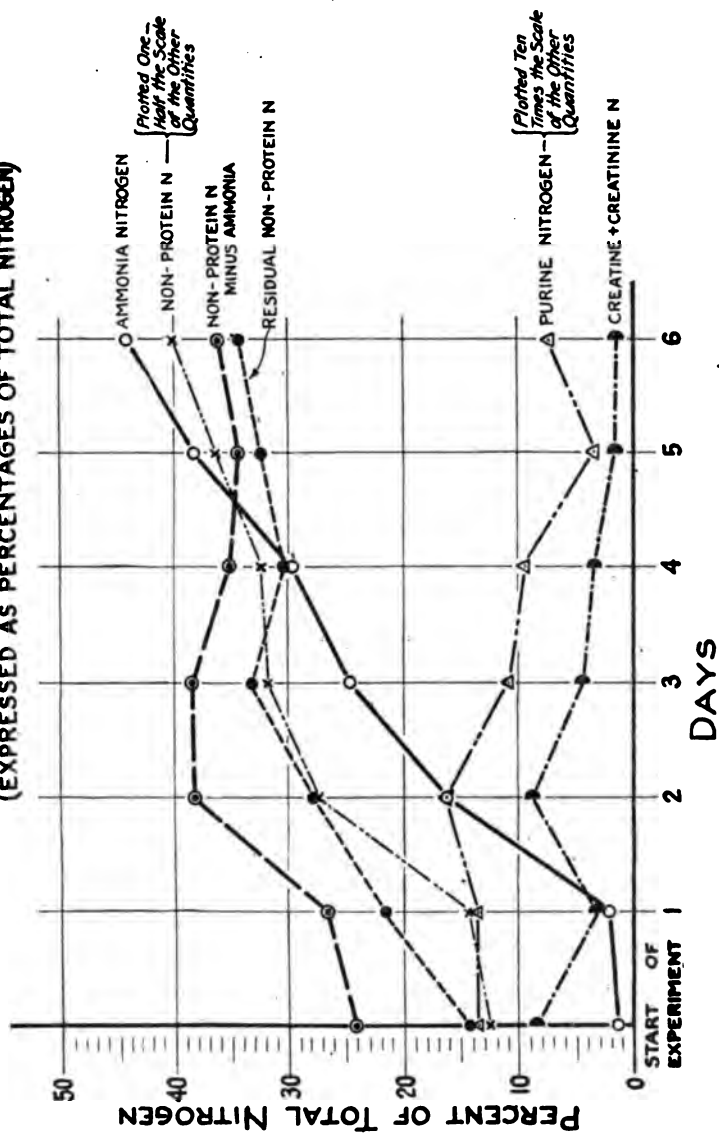
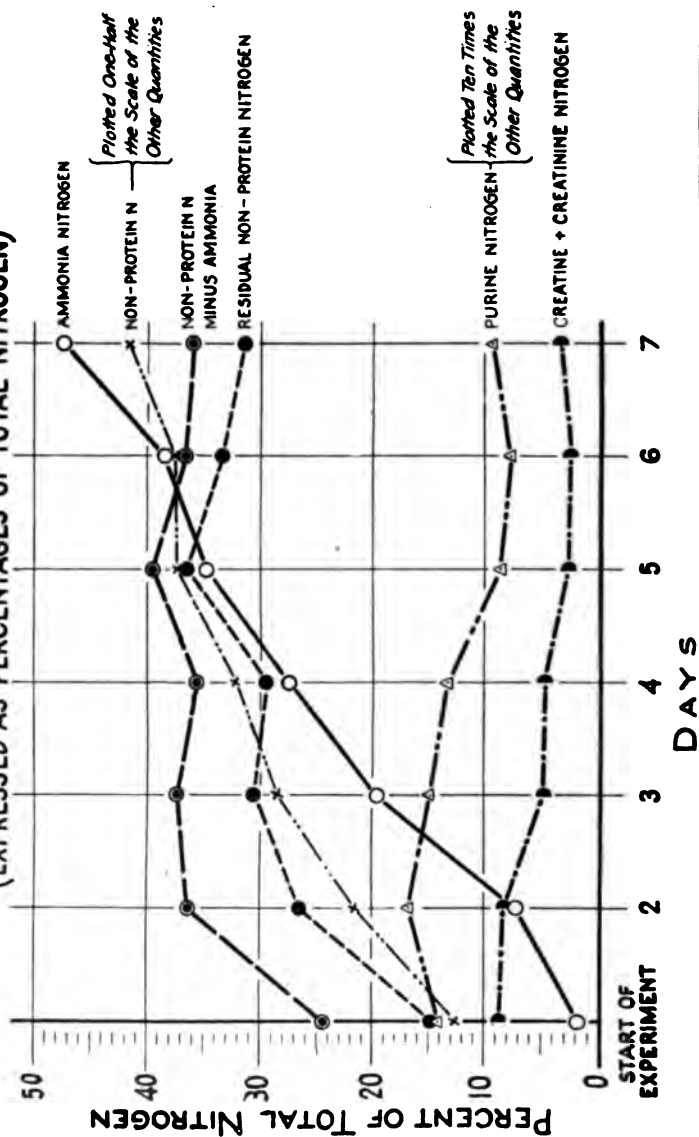


Fig. 8.

ACTION OF *STREPTOCOCCUS BREVIS* ON MEAT EXTRACT (EXPRESSED AS PERCENTAGES OF TOTAL NITROGEN)



AMMONIA TEST FOR MEAT SPOILAGE.

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(Received for publication, February 21, 1919.)

In the chapter on "Meat and meat products" in Allen's Commercial Organic Analysis,¹ Richardson stated in reviewing various tests that the estimation of the "nitrogen in ammonium salts, together with other substances easily decomposed by means of weak alkalis, probably affords the best available chemical methods at the present time for the detection of decomposition in flesh foods." Attention was directed to the difficulty of carrying out the methods because of the possibility of decomposition brought about by the reagents used. Ottolenghi, on the other hand, favored the determination of the amount of amino-acid as an index of the beginning of active decomposition.² Hoagland and associates³ found that the increase in amino nitrogen afforded the best measure of the extent of autolysis in cold-stored meats. The behavior of the ammoniacal nitrogen was much the same, but was not considered to be so good an index of the extent of the autolysis.

In the meat studies, some of the results of which were published in the preceding paper, the striking common factor in the decomposition of meat at room temperatures in the form of broth, brought about by a number of definite strains of bacteria, was the marked increase in the ammonia content, especially significant when calculated as percentages of total nitrogen or of non-protein nitrogen. Some experiments on amino-acid determinations showed

¹ Richardson, W. D., in Allen, A. H., Commercial organic analysis, Philadelphia, 4th edition, 1914, viii, 313-16.

² Ottolenghi, D., *Z. Untersuch. Nahrungs-u. Genussmittel*, 1913, xxvi, 728.

³ Hoagland, R., McBryde, C. N., and Powick, W. C., *U. S. Dept. Agric., Bull.* 433, 1917.

such small changes under the conditions used that this method was abandoned.

In attempting to find a chemical test for meat which would indicate when it was on the verge of being unfit to eat, the meaning of unfitness must be defined. Previous work on this question⁴ indicates that meat is not poisonous as food when thoroughly cooked if the animal was not infected before death. If the animal was infected when alive, the meat may contain heat-stable toxins. The test for unfitness for meat as purchased in this and other countries having suitable food laws must therefore be based upon its palatability when cooked or upon its flavor, odor, and appearance when uncooked. These standards will vary to some extent in different localities and with different individuals.

From the results of previous work, the ammonia content of meat undergoing decomposition under various conditions was chosen as the test and an attempt was made to find an approximate limit to the ammonia content when the meat would be considered unfit for food.

The difficulties in determining ammonia can be overcome by using the permutit method of Folin and Bell⁵ in which ammonia is extracted from a solution by specially prepared permutit (an aluminium silicate, zeolite). This ammonia is then liberated by treatment with alkali and determined by Nesslerization. The method eliminates the possible decomposition by alkali of the protein or other material present and gives results more truly representative of the ammonia in the meat present as such and as ammonium salts.

A number of series of determinations with the aeration and the permutit methods with fresh and decomposed meats gave similar results for the ammonia content, provided certain conditions were adhered to with the former. With 2 to 4 gm. of ground meat and 10 cc. of water, 1.25 cc. of 10 per cent NaOH solution was used, and aeration was continued for 2 hours. Caprylic alcohol was used to prevent foaming, 0.01 N sulfuric acid to absorb the ammonia, and 0.01 N sodium hydroxide for titrating, with the mixture of methyl red and methylene blue as indicator. Instead of sodium hydroxide, 10 cc. of 20 per cent sodium carbonate solu-

⁴ Greenwald, I., unpublished data.

⁵ Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

tion may be used, but aeration must then be continued for 3 to 4 hours. For the permutit method, 5 gm. of ground meat (beef) were treated with 60 cc. of water and 20 cc. of alumina cream in a 100 cc. volumetric flask for 1 hour, made up to the mark, filtered, and the ammonia was determined in the filtrate as described by Folin and Bell.

The results obtained with meat (beef) kept at room temperature and at low temperature showed marked differences. With the former, bacterial growth was rapid, especially at the higher temperatures, and the meat was soon unfit for use, in some cases after 24 hours. With the latter, there was considerable growth of mold with little bacterial growth. After trimming off the mold, the meat was still suitable for use after standing for 3 or 4 weeks at a temperature varying between 0° and 5°. With still lower temperatures, the meat could be used after much longer periods.

The ammonia content for the two forms of spoiled meat differed greatly. The fresh meat (beef within 24 hours of slaughter and chilled) contained between 0.03 and 0.10 mg. of ammonia N per gm. of meat. (The results will be given in these terms; the changes in moisture content of the meat were too small to affect the results.)

When allowed to decompose at room temperature (15–25°), the results of a large number of determinations showed that the meat became unsuitable for food when the ammonia nitrogen content reached 0.3 to 0.4 mg. per gm. of meat. Below this, the meat could still be used.

At low temperatures, the ammonia content was much higher before the meat was unsuitable to eat. The results varied considerably because of the variations in temperature and the previous condition and treatment of the meat, but the ammonia nitrogen content was over 1.0 mg. per gm. of meat in every case before the meat was unfit for use and in some cases rose to 3.0 mg. before the meat had to be discarded.

The interpretation of these results, especially in connection with the work of Hoagland and others, is simple. Bacterial growth at room temperature is comparatively rapid and the meat becomes unfit for food even with a low ammonia content. At low temperatures, bacterial growth is slow, but autolysis proceeds so that the cleavage products, such as ammonia and compounds rich in amino nitrogen, increase greatly without the formation of those products

whose odor, appearance, and general flavor make the food unsuitable for use. If the meat is kept cold first, autolysis proceeding, and then is brought to room temperature, decomposition would be much more rapid because of the simpler products formed by autolysis, which would serve as nutriment for bacteria and greatly increase their growth. This may be one reason, in addition to the physical effects of the breakdown of cell walls by freezing, for the more rapid decomposition of meat which has been in cold storage for some time.

The authors wish to express their thanks for the aid given by Miss Helen R. Downes and Mr. Robert P. Greene in carrying out the large number of analyses necessary in this work.

A SIMPLIFICATION OF THE MCLEAN-VAN SLYKE METHOD FOR DETERMINATION OF PLASMA CHLORIDES.

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(Received for publication, February 19, 1919.)

The method as originally published¹ required for each determination three filtrations as a preliminary to the titration; one after coagulation of the proteins with heat and magnesium sulfate, a second after treatment with charcoal to remove the last traces of protein, and a third after precipitation of the Cl with standard silver nitrate.

We have found that the three filtrations can be condensed into one by addition of picric acid to the standard silver nitrate. The technique consequently involves only one filtration and the measurement of an aliquot part of the filtrate for titration.

Rapplee² has recently published a method for plasma chloride determination, based on the Volhard titration, involving a technique as simple as ours, and in fact practically identical, viz. a precipitation with silver nitrate and titration of the filtrate, the difference being that Rapplee titrates the excess silver with sulfocyanate instead of iodide. We have obtained entirely accurate results with Rapplee's method. The choice between it and the iodide titration, therefore, depends upon individual preference.

Solutions.—The standard silver nitrate solution (Solution I, p. 363 of the original paper) is made up to contain, in addition to the silver nitrate and nitric acid, 7.5 gm. of picric acid per liter. This solution precipitates both proteins and chlorides simultaneously.³

¹ McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

² Rapplee, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

³ The silver nitrate solution contains per liter 5.812 gm. of pure fused AgNO_3 , 250 cc. of HNO_3 (specific gravity 1.42), and 7.5 gm. of picric acid.

The starch-citrate-nitrite solution contains per liter 446 gm. of crystalline sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 + 5\frac{1}{2} \text{H}_2\text{O}$), 20 gm. of NaNO_2 , and 2.5

The composition of the indicator solution, containing nitrite, citrate, and starch, is unchanged from that described in the original paper. It may be well to emphasize that when the starch is dissolved the solution must be not merely heated, but *boiled* for several minutes, or it will not give a satisfactory end-point. If starch other than the "soluble" variety is used the boiling should continue for an hour.

The $\frac{M}{58.5}$ KI solution, of which 1 cc. is equivalent to 1 mg. of NaCl, has been replaced by $\frac{M}{73.1}$ KI solution, of which 1 cc. is equivalent to 0.8 mg. of NaCl. The greater dilution of the KI solution is conducive to slightly increased accuracy in the titration; and the fact that it is equivalent to 0.8 mg. of NaCl per cc. simplifies the calculation to a single subtraction (see below). The KI solution is standardized to make 2.5 cc. equivalent to 1 cc. of the $AgNO_3$ solution.

Standardization of the KI Solution.—The solution contains 2.27 gm. of pure KI per liter. It is made up to contain 2.4 gm., and diluted to the extent indicated by a preliminary titration. 5 cc. of the silver nitrate solution, measured with a pipette which has an error not greater than 0.01 cc. (delivers 4.97 gm. of water at 20°), are mixed with 5 cc. of the starch-citrate solution and 5 cc. of water, and iodide is run in from a burette to a permanent blue end-point. The amount required should be 12.65 cc., 12.50 cc. being required to precipitate the standard silver solution, and 0.15 cc. additional to give the end-point (see below under "End-point").

Determination of Chlorides in Plasma.—2 cc. of oxalate or citrate plasma are drawn into a dry pipette calibrated to contain 2 ± 0.005 cc. (The pipette must weigh 1.994 ± 0.005 gm. more when filled with water at 20° than when empty and dry.) The plasma is run into a 50 cc. measuring flask half full of water, and the pipette is rinsed by drawing the water up into it twice. 10 cc. of the standard silver nitrate-picric acid solution are added, gm. of soluble starch. The starch is first dissolved in about 500 cc. of boiling water, to which the citrate and nitrite are then added, the solution being finally made up to 1 liter. 4 cc. of this solution contain sufficient citrate to react with the acid in 1 cc. of HNO_3 of 1.42 specific gravity, the resulting solution having the optimum acidity for production of the blue starch-iodine end-point.

and the mixture is diluted to the 50 cc. mark and shaken at intervals for several minutes, until coagulation is completed. The addition of a drop or two of caprylic alcohol prevents foaming and facilitates coagulation. The solution is passed through a dry, chloride-free filter, the first portion of filtrate being passed through, if necessary, a second time to remove turbidity completely. 20 cc. duplicate portions of the filtrate are measured with a calibrated pipette into 100 cc. Erlenmeyer flasks, 4 cc. of the starch-citrate indicator solution are added to each, and the standard KI is run in from a burette until a permanent blue end-point is obtained.

If it is desirable to use less than 2 cc. of plasma, 1 cc. measured within ± 0.002 cc. may be precipitated with 5 cc. of standard silver solution and diluted to 25 instead of 50 cc., the filtrate yielding only one 20 cc. portion instead of duplicates.

The End-Point.—Only a *permanent* and unmistakable blue is taken as the end-point. If the iodide is run in rapidly near the end of the titration, iodine may be formed more rapidly than the silver nitrate precipitates it, and a false end-point reached which disappears after a few seconds shaking. If, towards the end of the titration, the iodide is added in the usual manner, slowly, the blue shade caused by each drop disappears as soon as the solution is rotated, until the genuine end-point is reached. The latter is permanent, and in fact deepens with time.

The first appearance of the end-point is observed easily against a white background, but even more readily against one which matches the light yellow color of the silver iodide precipitate and the picric acid-tinged solution. We usually perform the titration over a sheet of light yellow paper which lies on the table beneath the burette.

The fact that 0.15 cc. of excess KI solution is required to give the end-point does not detract from the accuracy of the titration, because, with a given volume of solution, the required excess is constant and sharply defined. The end-point is not approached gradually, but appears suddenly on the addition of the last drop of the 0.15 cc. excess of KI. This may be demonstrated on a control solution by mixing 1 cc. of HNO_3 of 1.42 specific gravity with 4 cc. of starch-citrate indicator, diluting up to 25 cc., and titrating with the KI solution. The first 0.10 cc. produces no color at all, but the next drop suddenly causes a definite blue which deepens

rapidly for several seconds. The amount of excess KI required to produce the end-point varies directly as the volume of the solution; consequently it is desirable to keep the volume at the end of the titration within approximately the same limits (25 to 30 cc.) in standardizing as in performing the analyses.

Calculation.—The calculation is very simple when standard solutions of the above concentration are used. The 20 cc. of filtrate used for titration represent 0.8 cc. of plasma, and the unprecipitated portion of an amount of AgNO_3 equivalent to 8 mg. of NaCl, or 10 mg. per cc. of plasma. Each cc. of KI used in the titration is equivalent to 1 mg. of NaCl per cc. of plasma. Hence the calculation simplifies to:

$$\left. \begin{array}{l} \text{Mg. NaCl per cc. plasma, or} \\ \text{Gm. " " liter " } \end{array} \right\} = 10.15 - \text{cc. KI}$$

The use of 10.15 instead of 10 cc. is due to the fact that 0.15 cc. excess of KI solution is required to give the end-point. If the 0.15 cc. of excess iodide required to give the end-point were neglected in the calculation, the error would be partially eliminated by neglecting it also in the standardization. It is as simple, however, to allow for it in the calculation and thereby eliminate it entirely.

Necessity for Calibrated Apparatus.—As compared with most determinations used in physiological and clinical work, exceptional accuracy is required in the estimation of plasma chlorides, for the reason that the significant figure is not so much the total amount as the relatively small difference between that and the normal "chloride threshold" level of 5.62 gm. of NaCl per liter.⁴ Consequently errors of more than 0.10 gm. per liter are undesirable. In order to prevent such errors it is necessary to check the accuracy of all pipettes, burettes, and measuring flasks used by calibration. Glass measuring apparatus, because of slow shrinkage after blowing or error in original calibration, is not, as obtained from the dealers, sufficiently accurate to be used for this determination without being checked.

Comparison of Results with Those of Original Method.—The following parallel determinations on a series of human plasmas by

⁴ McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212, 366.

the original McLean-Van Slyke method and the present modification indicate that the results are identical.

Plasma No.	NaCl per liter.	
	McLean-Van Slyke method.	Present modification.
	<i>gm.</i>	<i>gm.</i>
1	5.90	5.90
	5.90	5.90
2	6.00	6.05
	6.04	6.02
3	6.00	6.00
	6.00	6.02
4	5.35	5.43
	5.35	5.38
5	5.35	5.40
	5.42	5.40
6	5.55	5.55
	5.50	5.52
7	5.68	5.65
	5.63	5.54
8	6.01	6.01
	6.01	6.03



THE NUTRITIVE VALUE OF THE WHEAT KERNEL AND ITS MILLING PRODUCTS.*

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

WITH THE COOPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

(Received for publication, February 20, 1919.)

INTRODUCTION.

In his review of the progress of physiological chemistry for 1917, Professor Hopkins¹ has remarked:

"The present shortage in the food supply of the world makes important every detail of knowledge concerning human nutrition. Even facts which seem academic need scrutiny, in case at some point or other they may find application in the direction of guidance for economy Particularly desirable just now is any scrap of knowledge concerning the cereals. Except in arctic climates, bread and cereals are always important items in the food of mankind, and except where wealth has accumulated and luxury come in its train, they are by far the most important. Circumstances have to be very exceptional indeed when the growing of cereals does not yield an energy supply for the worker at less cost and with less relative effort than any other method of food production. Economic and social factors usually tend to make bread by far the most convenient form in which the cereals can reach the individual consumer. The nations of the West have acquired the habit of demanding a well-piled loaf, and for this the special properties of wheat gluten seem necessary. Hence the reliance on wheat in the West."

It is not for mankind alone, however, that wheat has a nutritive importance; for this cereal seed and especially the by-products of its milling are largely used and highly esteemed in feeding domes-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ Hopkins, F. G., *Ann. Rep. on Progress of Chem. 1917, Chem. Soc. London*, 1918, xiv, 179.

tic animals. For reasons which need not be discussed here wheat has been milled more widely and has furnished a larger diversity of milling by-products than any of the other common cereals. These by-products have, in turn, acquired special uses in feeding practice so that their respective nutrient values are also worthy of independent consideration. The debate as to the relative value of the highly milled patent flour in contrast with graham flour or with "whole wheat" flour likewise hinges upon problems in comparative nutrition which have been uniquely emphasized by war time conditions.

Different Parts of the Wheat Kernel.

The wheat kernel consists of three chief parts, each anatomically and chemically differentiated from the others. These are *the embryo*, or *germ*, situated at one end of the kernel as a small, yellow mass, easily distinguished from the rest of the seed; *the endosperm*, which forms much the greater part of the entire kernel, and furnishes food for the embryonic plant when the seed germinates; *the outer seed coats* and underlying layer containing the protein cells which cover the entire seed and protect the embryo and endosperm from damage during the resting period of the seed's existence. Corresponding to the special functions of each of these three parts there are profound differences in the chemical nature of the substances composing them, and consequently wide differences in their nutritive value.

The accompanying tabulation gives the approximate proportion of the above named parts in the average moisture-free wheat kernel containing 2.2 per cent of nitrogen, equal to 12.5 per cent of protein ($N \times 5.7$); the proportion of the total protein of the seed in these parts; and also the percentage of the total protein in the part itself.

The relative proportion of these parts varies somewhat in different samples of wheat, but in general we believe that these figures fairly represent the average. The amount of endosperm was estimated from the difference between the sum of the other parts and the entire kernel. The amount of embryo was estimated by carefully dissecting out and weighing it. It is impossible to estimate accurately the amount of bran, for it cannot be completely separated from all the other parts of the seed by any method we have

discovered. From the results of milling tests on weighed quantities of wheat² and from the data given by Girard³ we have assumed that 15 per cent is not very far from the truth.

	Part as per cent of seed.	Per cent of seed as nitrogen in part.	Per cent of seed as protein in part (N \times 5.7).	Per cent of protein in part.	Per cent of total protein of seed in part.
Endosperm.....	83.5	1.61	9.17	11.1	73.3
Bran.....	15.0	0.49	2.80	18.7	22.3
Embryo.....	1.5	0.10	0.57	36.7	4.4
	100.0	2.20	12.54		100.0

Embryo.

This part of the seed is the center of its physiological activity, to the needs of which the other parts are subservient. Structurally the embryo consists of the rudimentary organs of the embryonic plant which are composed of a mass of small cells of which the nuclei form a far larger proportion than of any other part of the seed. In fact the cell nuclei are so abundant in the embryos that these and yeast have afforded the only material from which nucleic acid has as yet been obtained from vegetable sources.⁴ Under proper conditions of moisture and heat, the cells of the embryo begin to divide and chemical processes are set in operation which result in a rapid development of the new tissues of the seedling. The changes in structure, and therefore in composition, which constitute growth imply a high degree of instability in the chemical compounds which compose the greater part of the embryo. Owing to this latter fact chemical investigations of the embryo of wheat are particularly difficult and consequently our knowledge of its constituents is limited.

Frankfurt⁵ made a special investigation of the wheat embryo. From the data which he has furnished we have compiled the following statement of its chief constituents.

² Wiley, H. W., *U. S. Dept. Agric., Bureau of Chem., Bull. 13*, 1898, pt. 9, 1269.

³ Girard, A., *Compt. rend. Acad.*, 1897, cxxiv, 876, 926.

⁴ Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 379.

⁵ Frankfurt, S., *Landw. Versuchsstat.*, 1896, xlvii, 449.

Composition of the Wheat Embryo.

	<i>per cent</i>
Carbohydrates soluble in water, including sucrose and raffinose (6.89 per cent).....	24.34
"Crude protein" ($N = 6.44 \text{ per cent} \times 6.25$)*.....	40.25
Fiber.....	1.71
Crude fat.....	13.51
Ash.....	4.82
Undetermined (chiefly insoluble carbohydrates?).....	15.37
	100.00

* We have confirmed Frankfurt's estimate of nitrogen by Kjeldahl determinations made on very pure embryos isolated with great care.

Compared with the other parts of the seed the proportion of soluble carbohydrates is large, about 15 to 18 per cent of the embryo being sugars of which, according to Richardson and Crampton,⁶ sucrose is the most abundant, while another, supposed by them to be raffinose and later identified as such,⁷ is present in considerable amount. Starch is wholly lacking, as shown by microscopic examination of the isolated embryo.

Richardson and Crampton⁶ identified allantoin among the non-protein nitrogenous compounds and Frankfurt⁵ added to these asparagine, choline, and betaine. Later nucleic acid was found to be present in considerable, though undetermined, amount.⁴ In view of the fact that the proportion of these nitrogenous constituents is still unknown no accurate statement of the percentage of pure protein in the embryo can be given. It must, however, be decidedly less than the amount of "crude protein" as estimated by multiplying the total nitrogen by the conventional factor.

The proteins of the embryo differ markedly from those of the endosperm, both in their solubility and constitution.⁴ Water extracts about 10 per cent of albumin from commercial wheat embryo meal, and sodium chloride solution further extracts about 5 per cent of globulin, whereas only very small amounts of similar proteins are thus extracted from wheat flour (endosperm). It is probable that both albumin and globulin are present in larger proportion in the embryo than the above figures indicate, for the insoluble

⁶ Richardson, C., and Crampton, C. A., *Ber. chem. Ges.*, 1886, xix, 1180.

⁷ Schulze, E., and Frankfurt, S., *Ber. chem. Ges.*, 1894, xxvii, 64.

ble compounds which these proteins form with nucleic acid doubtless preclude their complete extraction. The proportion of the products of hydrolysis of the albumin, known as leucosin, is similar to that from proteins of many animal products which are commonly used for food, but is strikingly different from that yielded by the proteins of the endosperm of the wheat kernel.⁸

Owing to the difficulties encountered in separating the embryo from the other parts of the kernel it is practically impossible to obtain large quantities free from admixtures. We were therefore compelled to use a commercial preparation, kindly furnished by the Quaker Oats Company. By sorting this material under a lens about 33 per cent, obviously chiefly bran, could be separated, while the embryo tissue which remained still contained not a little endosperm as evidenced by the starch grains.

The following data, obtained by the conventional methods commonly used for analyses of this kind, show the general character of the material which we employed for our feeding experiments. From the nature of the methods employed these figures can at best represent only approximately the proportion of most of the designated groups of constituents.

Composition of Commercial Wheat Embryo Meal.

	<i>per cent</i>
Sugars.....	7.71
Dextrin.....	7.50
Starch.....	18.21
Pentosans, etc.....	8.29
Protein ($N \times 6.25$).....	31.00
Fiber.....	2.35
Fat.....	10.44
Ash.....	4.91
Undetermined.....	9.59
	<hr/> 100.00

By carefully grinding a sample of the meal after it had been extracted with ether the friable particles of the embryo were readily reduced to a powder while the more resistant particles of bran and a part of the endosperm were less easily reduced in size. By sifting on suitable sieves three fractions were obtained, the finest containing only a small amount of bran visible under the lens.

⁸ Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1906-07, xvii, 231.

The intermediate fraction, stained with iodine, consisted almost, if not wholly, of particles of endosperm and bran in approximately equal parts, so far as could be visually estimated. The coarsest fraction was chiefly bran to which some endosperm still adhered. Neither of the two coarser fractions appeared to contain appreciable amounts of embryo tissue. Assuming that one-half of the intermediate fraction was bran and that the endosperm in the coarsest fraction was approximately equal to the bran remaining in the finest fraction, we estimate that the original embryo meal before extraction with ether contained about 23.7 per cent of pure bran. From the starch content of the embryo meal we also estimate its content of endosperm at 23.5 per cent and in pure embryo tissue by difference at 52.8 per cent. Although the above method of determining the composition of the commercial embryo meal used for the experiments to be described later permits only a rough estimate, it gives the best idea of the character of this meal that we have been able to obtain.

The following tabulation shows the proportion of nitrogen and protein contained in this embryo meal, dried at 110°.

	Commercial embryo meal contains	
	Nitrogen.	Protein.
	<i>per cent</i>	<i>per cent</i>
Endosperm (N = 1.92 per cent) = 23.5	0.46 (N \times 5.70)	2.62
Bran (" = 2.88 " ") = 23.7	0.68 (" \times 6.25)	4.25
Embryo (" = 7.0 " ") = 52.8	3.70 (" \times 6.25)	23.10
Total N in meal.....	4.84	
Found.....	4.96	

The nitrogen and protein in this meal were in the following proportions:

	Nitrogen.	Protein
	<i>per cent</i>	<i>per cent</i>
In endosperm.....	9.5 (N \times 5.7)	8.7
" bran.....	14.1 (" \times 6.25)	14.2
" embryo.....	76.4 (" \times 6.25)	77.1
	100.0	100.0

Thus although only a little more than one-half of the embryo meal was probably pure embryo, more than three-quarters of the nitrogen belonged to that part of the seed.

Seed Coats or Bran.

Wheat bran is made up of several layers of cells which, when the seed is dry, adhere so firmly to one another that they are removed by the milling process in comparatively large pieces. By sifting and other mechanical means much is obtained nearly free from embryo and endosperm. As the separation is never perfect, however, even the purest commercial bran always contains a little endosperm and possibly traces of embryo.

The most conspicuous layer of cells composing bran is the innermost one which, in the entire seed, lies next to the endosperm. This layer does not belong strictly to the outer seed coats, for it is supposed to be a continuation of the embryo. For the purposes of our present study it must be included with the true seed coats because it is always removed with these latter in the milling process. The cells of this inner layer are thick-walled and contain a large part, perhaps the whole, of the protein of the bran. Unfortunately the thick walls of these cells make it so difficult to extract the proteins from bran that these have never been obtained in sufficient quantity or in suitable condition for determination of their physical or chemical characters. From the little that is known about these proteins, however, it is evident that they are unlike those of the gluten which constitute approximately 80 per cent of the protein of the endosperm. Chemically, as well as structurally, bran differs greatly from the embryo and endosperm and consequently also in its nutritive value. The thick cell walls which enclose so large a part of its nutrients render the digestion of these difficult for man, while cattle and poultry with their type of digestive organs utilize them readily.

As it is practically impossible to prepare large quantities of bran any freer from other parts of the seed than the so called pure bran of commerce, we have used a preparation of the latter for the feeding experiments described in this paper. The approximate proportion of the various groups of constituents contained in this preparation of bran is shown by the following data obtained by the

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same conventional method as was employed in analyzing the wheat embryo.

Composition of Commercial Bran, Moisture-Free.

	<i>per cent</i>
Sucrose.....	1.64
Dextrin.....	4.19
Starch.....	13.39
Pentosans, etc.....	27.58
Protein ($N \times 6.25$).....	17.00
Fiber.....	9.37
Fat.....	7.07
Ash.....	7.24
Undetermined.....	12.52
	<hr/> 100.00

Careful examination of this sample of bran showed that it was practically free from embryo. Since it contains starch it must consequently contain endosperm, for microscopic examination shows that none of the layers of cells which constitute really pure bran contains starch. The amount of endosperm in our commercial bran can, therefore, be estimated from its starch content at about 17 per cent. On this assumption the above analysis can be corrected to show more nearly the approximate composition of pure bran.

Approximate Composition of Pure Bran, Moisture-Free.

	<i>per cent</i>
Sucrose.....	1.92
Dextrin.....	5.03
Starch.....	0.00
Pentosans, etc.....	32.77
Protein ($N \times 6.25$).....	18.00
Fiber.....	11.18
Fat.....	8.26
Ash.....	8.61
Undetermined.....	14.23
	<hr/> 100.00

The proportion of nitrogen and protein in the commercial bran which we used was approximately as follows, calculated moisture-free:

	Commercial bran contains	
	Nitrogen.	Protein.
	<i>per cent</i>	<i>per cent</i>
Endosperm (N = 1.96 per cent).....	0.33 (N \times 5.7)	1.88
Bran (" = 18.0 " ").....	2.39 (" \times 6.25)	14.94
	2.72	16.82

Of the total protein in this preparation 11.2 per cent belonged to endosperm and 88.8 per cent to the pure bran.

Endosperm.

The endosperm forms about 84 per cent of the entire seed, the proportion varying with the plumpness of the grain. This part of the seed furnishes food for the growing embryo. Unlike the constituents of the embryo, those of the endosperm are relatively stable substances, designed by nature to remain unchanged until the germinating embryo draws on them for its first supply of food.

The following figures show the proportion of approximate constituents of the wheat flour which we used.

Composition of Wheat Flour, Moisture-Free.

	<i>per cent</i>
Sucrose.....	0.24
Dextrin, etc.....	3.63
Starch.....	77.52
Pentosans.....	1.80
Protein (N \times 5.7).....	11.44
Fiber.....	0.34
Fat.....	1.15
Ash.....	0.43
Undetermined.....	3.45
	100.00

In contrast to the other parts of the seed the endosperm is characterized by its very high starch content which, with the protein, equals nearly 89 per cent of the whole. Since most of the protein of the endosperm contains 17.5 per cent of nitrogen we have employed the factor 5.7 in estimating the percentage of protein from the nitrogen content of the flour.

The greater part of the total protein of the wheat kernel is stored in the large cells of the endosperm. Of this about 80 per cent consists of two kinds, gliadin and glutenin. These proteins unite to form the gluten, characteristic of wheat, which is physically and chemically unlike any protein product obtainable from other seeds. The unique physical properties of wheat gluten render flour made from this grain better for making bread than that obtained from the seeds of any other cereal. The tenacious character of wheat gluten when combined with water is such that the carbonic acid liberated by yeast when bread is leavened is so retained within the loaf as to impart to it a special lightness which cannot be secured by using any other flour.

Whole Wheat Kernel.

The proteins of the wheat kernel have been extensively studied in this laboratory⁹ and the knowledge thus gained, as well as that obtained in other laboratories, has been critically reviewed in a later publication.¹⁰ The relative proportion of amino-acids supplied by the wheat proteins is quite unlike that furnished by most other food proteins. Thus when hydrolyzed gliadin yields two and one-half times more ammonia, three or four times more glutaminic acid and proline, and only one-third to one-fourth as much basic amino nitrogen as do most food proteins. Furthermore, gliadin yields so little lysine¹¹ that young animals grow extremely slowly when fed on diets containing gliadin as the sole source of protein unless lysine is added to their food.¹² The products of hydrolysis of gliadin have been studied in this laboratory⁸ and more recent data were summarized later.¹³

Glutenin yields relatively large proportions of ammonia and glutaminic acid, though less than gliadin yields; also more basic amino-acids⁸ and enough lysine to permit of good growth when animals are fed on diets containing sufficient glutenin as the only

⁹ Osborne, T. B., and Voorhees, C. G., *Am. Chem. J.*, 1893, xv, 392.

¹⁰ Osborne, T. B., *Carnegie Institution of Washington, Publication* 84, 1907.

¹¹ Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd. M., *J. Biol. Chem.*, 1915, xxii, 259.

¹² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325.

¹³ Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 425.

protein.¹⁴ All of the amino-acid deficiencies of gliadin are, to some extent, supplemented by glutenin, so that wheat gluten affords a mixture of proteins on which young animals grow fairly well.¹⁵

Besides gliadin and glutenin the wheat endosperm (wheat flour) yields a small proportion of globulin, albumin, and proteose.⁹ Since similar proteins have been found in the embryo⁴ possibly their presence in flour may be due in part to an incomplete separation of the embryo in milling.

The relatively large proportion of ammonia, glutaminic acid, and proline yielded by the proteins of wheat flour compared with that yielded by proteins from other sources commonly used for food must have a marked influence on the nutritive value of white flour. Evidence of this is indicated by the experiments described in this paper, but further studies of this question should be made under more rigorously controlled conditions.

The accompanying figures give the proportion of the proximate constituents of the whole wheat used for most of the experiments described in this paper, as determined by an analysis which we have made according to the conventional methods employed for the analysis of the other parts of the kernel. The latter are here reproduced so that the very marked differences in their chemical composition may be the more readily appreciated.

	Whole wheat.	Endosperm.	Bran.	Embryo.
	per cent	per cent	per cent	per cent
Sucrose.....	0.61	0.24	1.92	24.34
Dextrin, etc.....	2.01	3.63	5.03	
Starch.....	63.80	77.52	0.00	0.00
Pentosans, etc.....	6.27	1.80	32.77	?
"Crude protein".....	11.25	11.17	17.60	40.25
Fiber.....	1.75	0.34	11.18	1.71
Fat.....	2.18	1.15	8.26	13.51
Ash.....	2.03	0.43	8.61	4.82
Undetermined.....	10.10	3.72	14.63	15.37
	100.00	100.00	100.00	100.00

¹⁴ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, pt. 2, 1911.

¹⁵ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxix, 69.

While we recognize that the above analyses may be fairly criticized on many grounds they serve to illustrate the very wide differences in the proportion of the various types of nutrients in the different parts of the wheat kernel into which it is separated by the miller.

Milling.

In the old fashioned process of milling, the entire kernel was ground between stones and the flour obtained by sifting through reels covered with fine-meshed silk cloth. Owing to the tough fibrous structure of the outer coats of the seed (bran) the greater part of this was removed, but a portion of the bran together with most of the embryo, or germ, passed through the sieve and was mixed with the flour. The product of this old fashioned milling was essentially the so called "entire wheat flour." When the bolting process was omitted and the coarse bran left in the ground product it has been known as graham flour.

Owing to the presence of the germ, flour made in this crude way cannot be kept long; and inasmuch as the bread made from such flour is dark in color the method of grinding wheat with stones has been almost entirely superseded by the Hungarian or roller mill process. The purpose of this is to separate the kernel into the three parts into which it is anatomically differentiated, namely the outer seed coats or bran, the embryo or germ, and the endosperm or berry of the grain. To accomplish this the grain is reduced to particles of successively smaller size; and by interposing suitable siftings on cloths of carefully chosen mesh the bran and germ are very completely separated from the flour. In carrying out this process about 30 per cent of the kernel is obtained, mostly as mixtures of all parts of the seed which cannot profitably be further separated.¹⁶ These mixtures are chiefly used for feeding domestic animals. It commonly has been assumed that high grade flour, from which bran and germ have been so completely removed, is less nutritious than that made by the older process; but little convincing evidence as to the justification for this assumption has been presented until recently.

¹⁶ A scheme representing the successive steps in the flour milling process has been published by Voegtlin, C., and Myers, C. N., *Public Health Rep.*, 1918, xxxiii, 911.

Nutritive Value of the Wheat Kernel and Its Parts.

Literature.—Although the nutritive value of the wheat kernel has been the subject of extensive investigations they have related almost wholly to the digestibility and utilization of its various parts and constituents. Since these studies have at present but little bearing on the subjects discussed in this paper we will limit our review to the more recent investigations directly concerned with the problems we have studied.

The *wheat kernel as a whole*, according to McCollum, Simmonds, and Pitz,¹⁷ contains sufficient water-soluble vitamine as well as protein of suitable quality to promote nearly normal growth in rats, provided the grain is supplemented with adequate salts and fat-soluble vitamine. In their experiments, however, reproduction was not normal until the protein of the ration was modified by the addition of casein.

The *wheat embryo*, according to the same authors,¹⁸ contains proteins of very superior quality, and an abundance of the water-soluble vitamine. The content of inorganic elements is inadequate and the amount of fat-soluble vitamine is insufficient to promote adequate growth when the embryo is depended upon to furnish these essential constituents of the ration. They also believe that the germ contains something which is distinctly toxic to animals, and can be partially removed by extraction with ether. With reference to this toxicity of wheat, Hopkins¹⁹ says:

“It is claimed that part of the symptoms displayed by animals on an exclusive wheat dietary containing the whole grain are due to a toxic factor. I am not quite sure that the evidence for this view is yet sufficient. Still further observations of a quantitative sort involving the adjustment of deficiencies seem first called for. Nevertheless, an oil with definitely toxic properties has been extracted from the wheat embryo.”

Likewise Voegtlin and Myers²⁰ assert that “no evidence of a toxic action of a whole wheat diet was obtained in the experi-

¹⁷ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 211. McCollum, E. V., and Davis, M., *ibid.*, 1915, xxi, 615.

¹⁸ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

¹⁹ Hopkins, F. G., *Ann. Rep. on Progress of Chem. 1917*, *Chem. Soc. London*, 1918, xiv, 180.

²⁰ Voegtlin, C., and Myers, C. N., *Public Health Rep.*, 1918, xxxiii, 843.

ments on squabs which were fed on whole wheat meal, supplemented by a suitable salt mixture." These investigators, speaking of the growth-promoting properties of foods derived from corn and wheat, arrived at the following conclusions:

"The 'highly milled' products are, without exception, inferior in dietary value, as regards growth, to foods prepared from the whole grain. It is rather surprising that such delicate organs as the gastro-intestinal tract of young mice can tolerate a diet containing a large amount of bran. This fact, however, does not necessarily mean that it is advantageous to include the bran in foods intended for human nutrition. On the contrary, the experiences with 'war bread' would rather indicate that persons with delicate digestion are subject to temporary digestive disturbances as a result of a change from 'white' bread to bread containing a considerable percentage of bran ('war bread'). On the other hand, from the standpoint of dietary completeness, a bread including all of the grain, with the exception of the superficial cellulose layer, is undoubtedly superior to the so-called white bread, made from 'highly milled' flour, and would not possess the above-mentioned objectionable features. The 'white' bread used in these experiments was not adequate for maintaining normal growth, in spite of the fact that it was prepared with some evaporated milk and yeast. The most significant defect of 'white' flour is the deficiency in antineuritic and fat-soluble vitamins; it is also deficient in adequate protein and inorganic salts. A wheat flour, containing a considerable part of the germ and superficial layers of the grain, supports growth of mice and pigeons especially well when supplemented with inorganic salts. The same is true of 'whole wheat' bread."

The nutritive value of the endosperm proteins, gliadin and glutenin, has been the subject of earlier reports by us.²¹ On otherwise adequate diets in which glutenin represents practically the sole source of protein, growth can readily be induced; not so, however, when gliadin, low in its yield of lysine, is similarly employed.

From the publications cited it appears that the water-soluble vitamins are chiefly, if not wholly, located in the embryo; that the proteins of the entire kernel, eaten in sufficient quantity, are adequate for normal growth; that the proteins of the embryo are superior to those of the endosperm in promoting growth; that the proteins of the endosperm are probably inferior in this respect to those of the entire kernel; and that better growth can be made when the proteins of the endosperm are supplemented by casein

²¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 473.

than when fed as the sole source of protein. However, owing to lack of data concerning food intakes only very general conclusions respecting the relative content in water-soluble vitamins, or of the nutritive value of the proteins of the endosperm and embryo of the wheat kernel, can be formed. Furthermore practically no information is available regarding wheat bran in respect to these nutritive factors.

Accordingly we have undertaken an extensive series of experiments on rats for the purpose of comparing quantitatively the entire wheat kernel and its milling products: wheat flour, wheat embryo, and wheat bran, with respect to the nutritive value of their proteins, and their content of water-soluble vitamins, both for the maintenance of adult rats and the growth of young ones.

EXPERIMENTAL.

Entire Wheat Kernel as a Source of Protein.

Maintenance Experiments.—Special difficulties are encountered in determining the protein minimum for maintenance, some of which we have discussed in a former paper.²² The additional experience gained in making the present experiments still further emphasizes these difficulties and has raised new questions which must be answered before we can draw very precise conclusions from the results obtained.

An inspection of the charts shows that protein intake and gain or loss of body weight do not run closely parallel, periods of slight gain in weight often being followed by a similar loss, although somewhat more protein was eaten in the latter period. Many cases where the converse is true are also to be observed. Occasional variations of 5 to 10 gm. in the weight of adult rats are of no importance in interpreting the results of these experiments, for such are to be attributed to more or less food, water, or feces in the animal at the time of weighing. A continued gain or loss, however, extending over several periods of weighing may be considered to represent a real change in the weight of the animal. The present series of experiments also has indicated that the vitamin concentration of the diet may have influence on the

²² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xxii, 241.

CHART I. The figures on the curves, indicating mg. of protein eaten weekly per gm. of body weight, show that when *whole wheat* furnishes all of the protein and water-soluble vitamins of an otherwise adequate dietary rats are not maintained unless their average weekly protein intake is above 20 mg. per gm. of body weight.

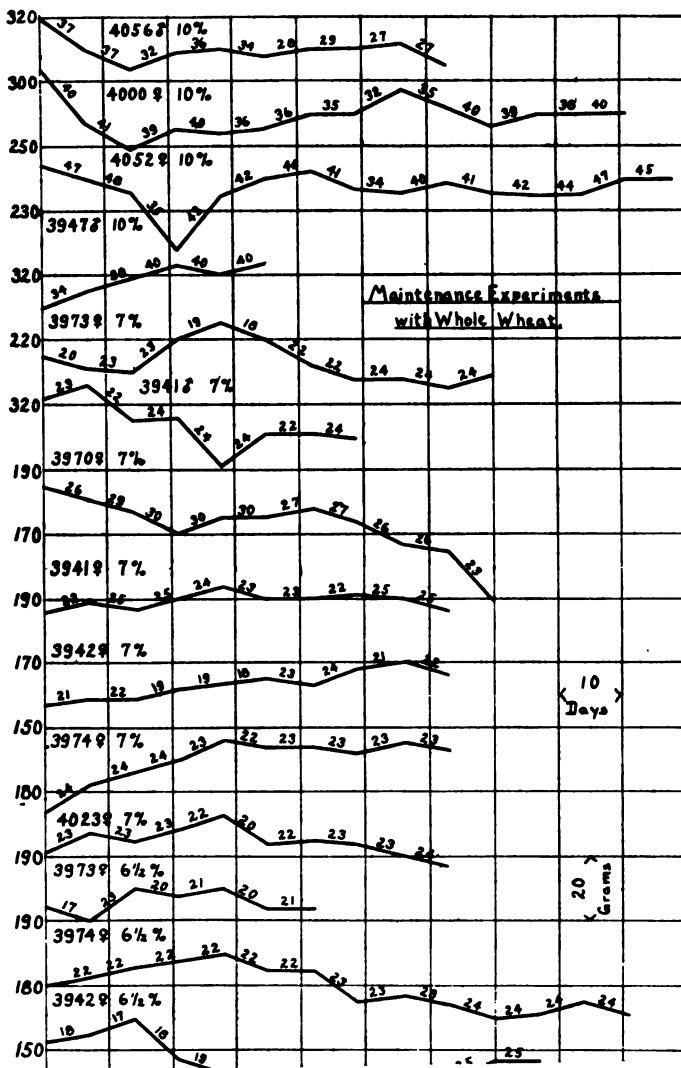
The composition of the food mixtures was as follows:

	5 per cent protein (N × 5.7).	6.5 per cent protein (N × 5.7).	7 per cent protein (N × 5.7).	10 per cent protein (N × 5.7).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole wheat.....	44	60	63	92
Salt mixture.....	3	3	3	3
Starch.....	28	12	9	
Butter fat.....	9	9	9	5
Lard.....	16	16	16	

The composition of the salt mixture was:

	<i>gm.</i>		<i>gm.</i>
CaCO ₃	134.8	Citric acid + H ₂ O.....	111.1
MgCO ₃	24.2	Fe citrate 1½ H ₂ O.....	6.34
Na ₂ CO ₃	34.2	KI.....	0.020
K ₂ CO ₃	141.3	MnSO ₄	0.079
H ₂ PO ₄	103.2	NaF.....	0.248
HCl.....	53.4	K ₂ Al ₂ (SO ₄) ₄	0.0245
H ₂ SO ₄	9.2		

The chemicals used were analyzed and allowance was made for moisture, etc. The acids were mixed and the carbonates and ferric citrate added to them. The traces of KI, MnSO₄, NaF, and K₂Al₂(SO₄)₄ were added as solutions of known concentrations. The final resulting mixture was evaporated to dryness in a current of air at 90–100°C., and ground to a fine powder.



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protein requirement; but concerning this we are not able to speak positively.

Rats were fed on foods containing 10 and 5 per cent of protein ($N \times 5.7$) furnished by whole wheat ground in the laboratory. In order to obtain a food containing as much as 10 per cent of protein, it was necessary to mix the ground wheat with salts, butter fat, and water and bake into cakes. The foods containing lower proportions of protein could be made into a coherent mass with an abundance of fat in the manner of our standard foods.

Chart I shows that when rats were fed *ad libitum* with the food containing 10 per cent of wheat protein they were well maintained for many weeks, although, for some reason not apparent, during the first 2 or 3 weeks a loss of weight followed the change from a mixed diet to the experimental ration. The amount of protein eaten (27 to 48 mg. per gm. of body weight) was thus found to be sufficient for maintenance. Another set of rats (see Chart I) supplied with food containing only 5 per cent of protein from the same whole wheat declined steadily in weight during many weeks, although they received all the food they would eat. These rats ate on the average about 16 mg. of protein per gm. of body weight. In order to show that the low content in protein, and not the total quantity of food eaten, was responsible for the falling weight, the same amount of food was fed daily, but containing 7 per cent of protein. Chart I shows that under this condition of feeding body weight was well maintained and that an intake of about 23 mg. of the total proteins per gm. of body weight per week is sufficient for maintenance during relatively long periods. From the results of these experiments we conclude that less than 20 mg. of the total wheat protein are insufficient for prolonged maintenance of most adult rats and that in general about 23 mg. are sufficient.

These figures show that for maintenance the proteins of the wheat kernel are not greatly inferior to casein, edestin, or even to the total proteins of milk, but are somewhat superior to gliadin.²²

Growth Experiments.—Owing to the low protein content of wheat kernel used for our experiments²³ it was impossible to prepare a food comparable to our earlier mixtures in fat content and calorific

²² Owing to the shortage of wheat we were compelled to use such samples of wheat and flour as we could obtain locally.

value, and still containing more than 8 per cent of protein. Diets with lower contents furnish too little protein for normal growth. Accordingly a food was prepared containing 92 per cent of the whole wheat kernel ground in the laboratory (= ca. 10 per cent protein, $N \times 5.7$) supplemented with salts and only 5 per cent of butter fat. This was mixed with enough water to make a soft dough and fed either in the moist form, or after drying in hard cakes. In some cases, the unground kernels were smeared with the mixture of butter fat and salts and fed whole. The last method was the least satisfactory, because some of the rats at times ate the embryos, leaving a part of the bran and endosperm uneaten, so that their diet was not strictly representative of the entire grain. As these foods were much lower in calorific value than our ordinary mixtures containing 25 to 30 per cent of fat, the rats ate about 50 per cent more of these rations than of our "standard" foods, and thus actually consumed as much total protein as rats on our ordinary fat-rich diets which contain 15 to 17 per cent protein. This is a further illustration of how misleading may be conclusions based on the *percentage* of protein in the food when nothing is known regarding the *amount* of food and protein *eaten*.²⁴

These experiments, for which graphic records are shown in Chart II, demonstrate that the wheat proteins considered in their entirety are adequate for promoting normal growth if eaten in sufficient amount.

We can thus substantiate the evidence that the proteins of the entire wheat kernel suffice to promote the growth of rats to normal adult size. The quantity of protein required is, however, relatively large.

Wheat Embryo as a Source of Protein.

The embryo constitutes about 1.5 per cent of the entire kernel. The protein value of the embryo comes into prominence in milling by-products, although from a *protein* standpoint when the whole wheat is used the embryo can play a small rôle at best. Commercial wheat embryo meal contains relatively large amounts of both bran and endosperm, the proportions often being quite

²⁴ This point has been discussed elsewhere; see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 521.



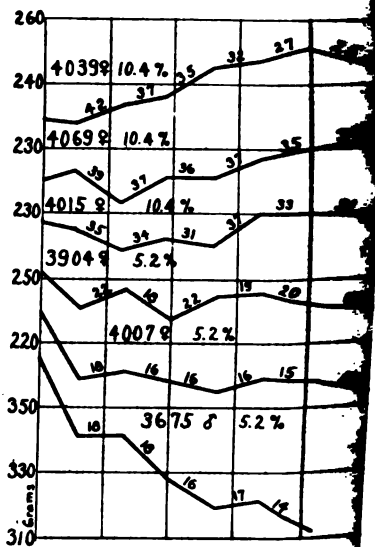


CHART III. The figures of Rats are maintained on diets by commercial wheat embryo. Rats 3904 and 4007 were of body weight. Rat 3675 The composition of the

Wheat embryo.
Salt mixture*...
Starch.....
Butter fat.....
Lard.....

* See legend,

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unlike in different samples. The preparation used for these experiments, which contained about 50 per cent only of the pure embryo has already been described on page 561. Although preparations containing a larger proportion of embryo are obtainable, such were not available at the time these experiments were undertaken. From the results of our analyses of this preparation it appears that about 77 per cent of the nitrogen in the commercial embryo meal used for the experiments here described belongs to the pure embryo.

Maintenance Experiments.—Adult rats have been well maintained for several weeks on a diet containing about 10.4 per cent of “crude protein” ($N \times 6.25$) derived entirely from commercial wheat embryo meal equivalent to about 8.1 per cent of “crude protein” from the pure embryo (see Chart III). The quantities of food which these animals ate gave them a weekly protein intake of 24 to 50 mg. per gm. of body weight. That these quantities are above the minimum for maintenance is shown in Chart III by Rat 3904 ♀ which was maintained for 5 weeks on an average of 20 mg., and by Rat 4007 ♀, maintained for 6 weeks on about 16 mg. of the commercial embryo protein per week, the “crude protein” content of their rations being 5.2 per cent. Both of these rats, however, declined slightly, during the period of feeding as a whole, and Rat 3675 ♂ declined rapidly, although the protein intake per gm. of body weight was quite as large as that of Rat 4007. Why this rat declined is not clear, especially as subsequently it recovered promptly on a mixed diet.

Our evidence is scarcely sufficient to justify final conclusions as to the minimum quantity of embryo protein required for maintenance. However, the data indicate that the “crude protein” in this commercial embryo meal was more efficient for maintenance than is that of the entire wheat kernel.

Growth Experiments.—The proteins of the wheat embryo are much more efficient than those of the entire kernel for promoting growth. Diets containing as little as 7.0 per cent of “crude protein” ($N \times 6.25$) of which 77.1 per cent was from the embryo, 14.2 per cent from bran, and 8.7 per cent from endosperm, induced considerable growth in rats (Chart IV, Rats 4580 ♂, 4571 ♂, 4587 ♀, 4573 ♀, Period 1); and when the protein amounted to 13.7 per cent the animals made practically normal growth during

several months (Chart IV, Rats 4621 ♀, 4601 ♂, 4608 ♂, 4604 ♀, and Rats 4580 ♂, 4571 ♂, 4587 ♀, 4573 ♀, Period 2).

Table I shows the growth-promoting value of the proteins of the embryo of wheat in comparison with those of wheat bran,

TABLE I.
Comparisons of Gains of Body Weight per Gm. of Ingested Protein When Furnished by Wheat Embryo, Wheat Bran, Casein, or Lactalbumin.

Source of protein.	Protein in food.	Rat.	Initial body wt.		Gain in 4 wks.		Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
							Food.	Protein.	Food.	Protein.	
	per cent		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Wheat embryo.....	13.7	4621 ♀	60	41	208	28.5	5.1	0.69	1.44		
		4608 ♂	74	52	232	31.8	4.5	0.61	1.63		
		4604 ♀	59	42	204	28.0	4.9	0.67	1.50		
		4601 ♂	59	56	225	30.8	4.0	0.55	1.82		
Average.....							4.6	0.63	1.60		
Wheat embryo.....	6.95	4571 ♂	64	21	206	14.3	9.8	0.68	1.47		
		4573 ♀	67	25	220	15.3	8.8	0.61	1.63		
		4580 ♂	62	30	243	16.9	8.1	0.56	1.78		
		4587 ♀	70	31	282	19.6	9.1	0.63	1.59		
Average.....							9.0	0.62	1.61		
Wheat bran.....	9.05	4635 ♂	67	44	280	21.5*	6.4	0.49	2.06		
Casein.....	11.0	2117 ♂	69	38	159?	17.5?	4.2	0.46	2.17		
		2623 ♂	65	62	217	23.8	3.5	0.38	2.60		
		2630 ♂	63	67	233	25.6	3.5	0.38	2.61		
Average.....							3.6	0.41	2.46		
Lactalbumin.....	10.3	2472 ♂	62	37	166	17.1	4.5	0.46	2.16		
		2474 ♂	71	60	213	21.9	3.6	0.37	2.74		
		2931 ♂	63	47	189	19.5	4.0	0.42	2.41		
Average.....							4.0	0.41	2.44		

* 15 per cent deducted on account of poor utilization of this food.

milk casein, or lactalbumin, established according to the method recently developed in this laboratory.²⁵ The average amount

²⁵ Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.



of gain per gm. of protein eaten during 4 weeks was 1.60 gm. In these experiments growth was obviously determined by the amount of protein eaten; hence this figure may be accepted as a fair numerical expression of the value of the embryo proteins for growth, and shows that the "crude protein" of the embryo is more efficient than that of the entire kernel and decidedly more so than that of the endosperm or wheat flour (see Tables II and III).

Wheat Bran as a Source of Protein.

Growth Experiments.—On a diet containing 55 per cent of commercial wheat bran, which furnished 9 per cent of protein ($N \times 6.25$), 88.5 per cent of which was from bran and 11.5 per cent from endosperm, one rat grew at a nearly normal rate for several weeks, although three other animals on the same diet grew very little. Analysis of the feces of these four rats showed that 75 to 80 per cent of the total food and 70 to 75 per cent of the nitrogen was utilized. The one rat which grew well ate very liberal quantities of the food, proving that the "crude protein" in bran is adequate for growth if enough is eaten (see Chart IV, Rat 4635).

The data given in Table I show that the "crude protein" of wheat bran has a higher value for the growing animal than does that of the embryo. This one rat, which grew well on a diet containing 9.05 per cent of "crude protein" ($N \times 6.25$) from bran gained 2.05 gm. in body weight per gm. of "crude protein" eaten. The "crude protein" of bran appears to be quite as efficient as that of the combination of wheat flour with egg, milk, or meat, under the conditions of this experiment (see Table II).

If we assume, as is almost certainly the case, that the proportion of *pure* protein in bran is less than that of the "crude protein," as estimated by multiplying the nitrogen by the conventional factor 6.25, the efficiency of bran protein, if it could be isolated and tested in the pure state, would unquestionably be found higher than that of the "crude protein." It must not be forgotten that our estimate of the value of the latter is based on a single experiment, and that we have offered no evidence to

prove that the maximum efficiency of the "crude bran protein" has been finally established. The concentration of protein in the bran diet fed, however, was so low that probably growth which was not quite normal, was limited by the amount of protein ingested. These data indicate that the agriculturist is justified in his high estimate of the value of wheat bran as a protein concentrate.

Wheat Endosperm as a Source of Protein.

For this purpose wheat flour (Famous XXX brand) containing, air-dry, 1.43 per cent N equal to 8.15 per cent protein ($N \times 5.7$) was used. Efforts to obtain flour of higher protein content failed owing to the restrictions imposed on milling which prevailed at the time these experiments were begun. Since this flour was so low in protein, it was necessary, in most of the experiments, to increase the protein by adding wheat gluten, made by washing the starch out of the same lot of flour. The results obtained were presumably not seriously affected by this procedure because upwards of 80 per cent of the nitrogen of the endosperm was recovered in the gluten used.

Maintenance Experiments.—On diets containing about 7.5 per cent of protein, two-thirds from the wheat flour and one-third from gluten, all but one of these adult rats were very nearly maintained for many weeks (see Chart V). These animals ate on the average 23 to 26 mg. of protein per gm. of body weight per week, quantities essentially like those found to be adequate for maintenance on the entire kernel. One rat, No. 3932♂, steadily declined in weight on this intake until the protein of the gluten was replaced by an equivalent amount of meat residue, the total food intake being unchanged. 4 weeks later this rat was killed and found to have infected lungs. Whether or not this disease was the cause of its higher protein requirement cannot, of course, be determined. The addition of 0.1 gm. of wheat embryo daily (Rats 3983♀, 3953♀) caused the animals to be better maintained although their food intake was restricted to the quantity eaten during the previous period and their weekly protein intake, about 25 mg. per gm. of body weight, was practically unchanged. When the gluten was omitted and the endosperm diets contained only about 5 per cent of protein, giving the animals an average

weekly protein intake of about 16 mg. per gm. of body weight, all declined steadily, showing that for the protein of the wheat endosperm this quantity is too low. The *total* food intake during the period of decline had previously been found to be adequate for maintenance. When 1 per cent of protein as meat residue replaced an equal quantity of wheat flour proteins in this 5 per cent protein diet of the rats which were declining, and a small amount of commercial wheat embryo was fed, to compensate for the diminution of water-soluble vitamine due to replacing 20 per cent of the flour in the food with starch, the animals were maintained better, although their food intake was kept unchanged. Their weekly protein intake, including the small amount furnished by the wheat embryo, averaged about 18 mg. per gm. of body weight. These experiments, together with that on Rat 3932 in the period during which it had meat protein but no added embryo, indicate that when a suitable protein is used to supplement the amino-acid deficiencies of the wheat proteins *less* protein is required for maintenance than when wheat flour is the sole source of protein.

Growth Experiments.—Young rats have made almost no growth on diets all the protein of which was furnished by wheat flour, in spite of the fact that they consumed as much food, as well as protein per week, as the majority of rats of comparable size which have grown well on our ordinary rations, similar in calorific value and protein content. When fed on these wheat flour diets rats whose final average body weight was 81 gm. ate on the average 37 gm. per week, whereas the majority of normally growing animals of 80 gm. of body weight eat weekly about 38 gm. of food of like calorific value.²⁶ Hence we can conclude that the failure of these rats to grow (see Chart VI, Series A) was not due to insufficient food intake. The failure to grow therefore may have been due to either a deficiency of vitamine, to poor quality of the flour proteins, or to both of these factors combined.

Experiments with this flour as the sole source of water-soluble vitamine in otherwise adequate diets have led us to conclude that even 60 per cent furnished less than the optimum amount of vitamine for growth (see p. 591). We accordingly increased the amount of vitamine supplied to another series of rats (Chart VI,

²⁶ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

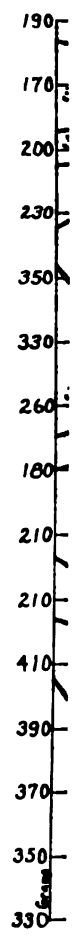
CHART V. The figures on the curves show that a weekly protein intake of 23 to 26 mg. per gm. of body weight barely suffices to maintain adult rats when both protein and water-soluble vitamins are furnished solely by *wheat endosperm* (flour). When the water-soluble vitamins were increased by feeding a little commercial wheat embryo separately and the quality of the protein improved by replacing one-fifth of the endosperm protein by meat protein, Rat 3979 was maintained on an average weekly protein intake of 16 mg. per gm. of body weight. The proteins of flour are more economically utilized in a mixed diet containing meat and supplying sufficient water-soluble vitamins. Rat 3932 shows the effect of improving the quality of the protein without changing the vitamin content of the ration. The failure of this rat to be maintained on a protein intake equal to that of Rats 3983, 3953, 3974, and 3973 may possibly be due to higher vitamin requirement owing to its relatively large size. That this may be so is indicated by other experiments which we are now making.

The composition of the food mixtures was as follows:

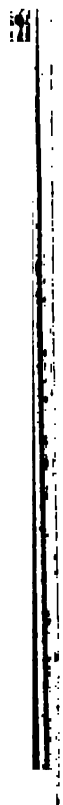
	5 per cent protein (N \times 5.7).		7.5 per cent protein (N \times 5.7).	
	Period 1. Rats 3979, 3972, 3971, 3942, 3947	Period 2. Rats 3979, 3972, 3971	Period 1. Rats 3983, 3953, 3974, 3973, 3932	Period 2. Rat 3932 Period 3. Rats 3979, 3972, 3971
	per cent	per cent	per cent	per cent
Wheat flour.....	62.0	50.0	62.0	60.0
" gluten.....			3.3	
Meat residue*.....		1.2		3.0
Salt mixture†.....	3.0	4.0	3.0	3.0
Starch.....	10.0	19.8	6.7	9.0
Butter fat.....	9.0	9.0	9.0	9.0
Lard.....	16.0	16.0	16.0	16.0

* See Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 313.

† See legend, Chart I.



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Series B) which were otherwise fed on the same dietary as Series A, by giving them separately as much of the solids of a protein-free aqueous extract of dried yeast as had been proved by other experiments to furnish enough vitamine for nearly normal growth. This yeast fraction was equal to 16 per cent of the original yeast and the amount eaten contained nitrogen equal to protein equivalent to 1.6 per cent of the average amount of protein ingested during the course of these experiments. In spite of this large increase in the vitamine consumed and also of a possible supplementing of the flour protein by the nitrogenous constituents of the yeast extract these rats grew little if any better than those of Series A.

When the food contains adequate protein, but is deficient in vitamine, satisfactory growth always follows an addition of a substantial quantity of vitamine; hence we must conclude that the rats shown in Chart VI, Series A, failed to grow because the proteins of the wheat endosperm flour were inadequate, even when these amounted to 15 per cent of the diet.

Since wheat flour is rarely the sole source of protein in the human dietary it is quite as important to know the nutritive value of the protein mixtures resulting from the combination of flour with the various food products which are commonly eaten with bread, as to know that of the proteins of bread alone. Having learned from the experiments already described that the proteins of the wheat endosperm are adequate for maintenance, but inadequate for normal growth, even when liberal quantities are eaten, we next attempted to compare the rate of growth made on the proteins of flour alone with that made on corresponding quantities of protein, two-thirds of which was supplied by wheat flour and one-third by egg, milk, or meat, together with vitamine in presumably adequate quantity. Egg powder and whole milk powder (Merrell Soule) were used to furnish the proteins from egg and milk. The meat was furnished by lean round of beef which was dried and ground to a powder in the laboratory.²⁷ It was considered, in view of our other numerous experiments, that the quantity of these animal products, in conjunction with the 55 to 60 per cent of wheat flour included in these diets, would fur-

²⁷ Cf. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 313.

nish an adequate amount of the water-soluble vitamin. Sufficient fat-soluble vitamin was provided for by liberal additions of butter fat.

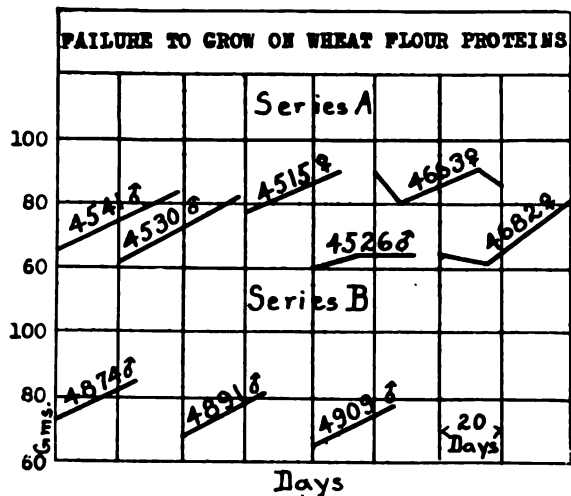


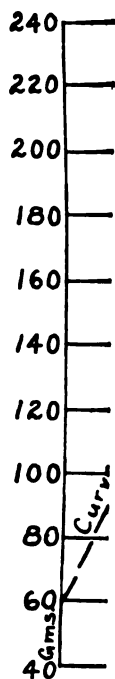
CHART VI. When wheat *endosperm* (flour + wheat gluten) furnishes the sole source of protein rats fail to grow (Series A). Scarcely any improvement results when the vitamin content of the food is increased by addition of a water extract of yeast (Series B).

The composition of the food mixtures was as follows:

	Series A.	Series B.
	per cent	per cent
Wheat flour.....	58	58
“ gluten.....	13	13
Salt mixture.*.....	4	4
Butter fat.....	9	9
Lard.....	16	16
Yeast extract.....		0.2 gm. per day

* See legend, Chart I.

The results obtained with these diets, each of which contained nitrogen equal to 14.8 per cent of protein, are shown in Chart VII, Periods 1 and 4. When the supplementary proteins were re-



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placed by an equivalent quantity of wheat gluten, Period 2, the growth of the animals was checked almost instantly. Since in Period 2 flour was the sole source of the water-soluble vitamins an additional amount was furnished during Period 3, in the form of an aqueous extract of yeast. The rate of growth and food intake in most cases was increased thereby over that in Period 2, but even in Period 3 no rat grew even approximately as rapidly as in Periods 1 and 4 in which the superior combination of proteins was fed.

These results show plainly that the supplements—egg, milk, or meat—enhance the value of wheat flour as a source of protein for the growing animal.

Since in Periods 1 and 4 growth was at the normal rate, protein may have been wasted through being consumed in greater quantity than was actually needed. Under such circumstances if differences in supplementing value exist between eggs, milk, or meat these would not be revealed. Accordingly another series of experiments was made using similar diets but containing only 10.3 per cent of protein, a proportion supposedly low enough to limit growth to less than the normal rate. Inasmuch as growth was so slow on the diet containing 14.8 per cent of protein derived wholly from the wheat flour it seems fair to assume that on this diet growth was at the maximum rate possible with this proportion of protein.

When egg or milk powders were used to supplement the flour proteins no other source of water-soluble vitamins was deemed necessary. Since flour and meat powder are low in vitamins two series of experiments were made with the meat supplement, one with, the other without, extra vitamins supplied by 0.2 gm. of dried yeast fed separately each day. The fat-soluble vitamins were supplied by butter fat, allowance being made for the fat in the egg or milk powder, which was assumed to have a value equal to that of butter fat as a source of this factor. Additions of lard made the total amount of fat in each food 25 per cent, consequently the total calorific value of the several diets was practically identical. The results of these experiments are shown in Table II, from which it is seen that decidedly more rapid growth occurred when the diet contained only 10.3 per cent of protein, furnished by flour supplemented with egg, milk, or meat, than when it con-

tained even 50 per cent more protein derived entirely from the wheat flour.

The following summary shows the average relative gains of body weight, per gm. of protein eaten, made by the rats fed on these different diets.

	Per cent of protein in food.	Gain of body weight per gm. of protein.
		gm.
Flour + egg.....	14.8	2.00
	10.3	1.80
“ + milk.....	14.8	1.67
	10.3	1.73
“ + meat.....	14.8	1.73
	10.3	1.47
“ + “ + yeast.....	10.3	1.66
“ + gluten.....	14.8	0.50

For the data from which these figures were derived see Table II. From them it appears that eggs or milk supplement flour proteins slightly more efficiently than does meat even though additional vitamine was supplied by yeast.

The fact that in the experiments with 10.3 per cent of protein the total gain of weight was so much less than with 14.8 per cent shows that in the former case protein was the limiting factor. Since the gains per gm. of protein eaten were so nearly alike for the two concentrations of protein, we are justified in the assumption that the higher figures represent approximately the maximum efficiency of these protein mixtures for promoting growth. We realize that such a mathematical expression of the relative merits of these food mixtures is not strictly justifiable, because if growth on the flour proteins alone had been sufficiently slow its amount, stated in gm., would approach zero, at which point the difference between the amounts of protein required would equal infinity when flour protein alone is contrasted with its combinations with the animal proteins.

Having thus obtained evidence of the effect on the nutritive

TABLE II.
(See Chart VII.)

Gain of Body Weight Per Gm. of Ingested Protein Furnished by Wheat Endosperm (Flour) Combined with Egg, Milk, or Meat, and by Endosperm Alone.

Source of protein.	Protein in food.	Rat.	Initial body wt.	Gain in 4 wks.	Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
					Food.	Protein.	Food.	Protein.	
	per cent		gm.	gm.	calories*	gm.	calories*	gm.	gm.
Wheat flour } " gluten }	14.8	4676 ♂	69	62	1,260	33.8	20.4	0.55	1.83
Egg powder }		4684 ♂	54	82	1,335	35.9	16.3	0.44	2.28
		4688 ♂	60	76	1,335	35.9	17.6	0.47	2.12
		4679 ♀	69	61	1,240	33.4	20.4	0.55	1.83
Average.....							18.7	0.50	2.02
Wheat flour } " gluten }	10.3	5102 ♂	67	49	1,410	25.0	28.8	0.51	1.96
Egg powder }		5115 ♂	70	49	1,570	27.9	32.0	0.57	1.76
		5111 ♂	72	43	1,430	25.4	33.2	0.59	1.69
		5104 ♂	65	40	1,270	22.6	31.8	0.57	1.77
Average.....							31.5	0.56	1.80
Wheat flour } " gluten }	14.8	4673 ♂	70	70	1,240	38.7	17.7	0.55	1.81
Milk powder }		4675 ♂	62	49	1,050	32.8	21.4	0.67	1.49
		4690 ♂	56	78	1,200	37.4	15.4	0.48	2.08
		4678 ♀	75	61	1,335	41.6	21.8	0.68	1.47
Average.....							19.1	0.60	1.71
Wheat flour } " gluten }	10.3	5120 ♂	71	54	1,645	29.8	30.4	0.55	1.81
Milk powder }		5121 ♂	71	44	1,410	25.4	32.1	0.58	1.73
		5128 ♂	70	40	1,345	24.3	33.6	0.61	1.65
		5191 ♂	72	62	1,430	25.8	23.1	0.42	2.40
Average.....							29.8	0.54	1.90
Wheat flour } " gluten }	14.9	4670 ♂	75	69	1,260	38.2	18.2	0.55	1.81
Meat powder }		4672 ♂	65	60	1,070	32.5	17.8	0.52	1.85
		4686 ♂	68	56	1,180	35.8	21.0	0.64	1.56
		4677 ♀	65	47	940	28.6	20.0	0.61	1.64
Average.....							19.3	0.58	1.72

* Estimated (protein + carbohydrates \times 4.1, fat \times 9.3 calories).

TABLE II.—*Concluded.*

Source of protein.	Protein in food.	Rat.	Initial body wt.		Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
	per cent		gm.	gm.	Food. calories*	Protein. gm.	Food. calories*	Protein. gm.	
Wheat flour	10.3	5116 ♂	70	31	1,190	22.4	38.4	0.72	1.38
“ gluten		5129 ♂	72	28	1,000	18.8	35.8	0.67	1.49
Meat powder		5117 ♀	66	38	1,310	24.5	34.5	0.64	1.55
Average.....							36.2	0.68	1.47
Wheat flour	10.3	5236 ♂	72	31	1,070	19.9	34.5	0.64	1.56
“ gluten		5245 ♂	73	37	1,095	20.4	29.6	0.55	1.82
Meat powder + 0.1 gm. yeast daily		5246 ♂	75	33	1,220	22.6	37.0	0.68	1.46
		5249 ♂	76	42	1,270	23.4	30.2	0.56	1.80
Average.....							32.8	0.61	1.66
Wheat flour	14.7	4874 ♂	75	11	860	25.3	78.1	2.30	0.43
“ gluten + 0.2 gm. yeast extract daily		4891 ♂	68	14	915	26.9	65.4	1.92	0.52
		4909 ♂	65	13	805	23.7	62.0	1.82	0.55
Average.....							68.5	2.01	0.50

* Estimated (protein + carbohydrates \times 4.1, fat \times 9.3 calories).

value of wheat flour caused by combining it with those animal products most generally eaten together with bread, it remains to be determined whether equally good results may not be secured by leaving in the flour those parts which are customarily separated from it by the miller and subsequently used to supplement the protein deficiencies of the rations of farm animals. In making experiments to decide this question a little yeast was added to the diet because our earlier experience showed that the endosperm, bran, and embryo, fed alone in the amounts used, supplied too little vitamine for normal growth. Although the proteins of the yeast undoubtedly supplement those of the wheat, nevertheless we believe the results of these experiments are comparable with one another, as well as with those obtained with foods containing a like percentage; namely, 10.3 per cent of protein derived from whole wheat or combinations of flour with eggs, milk, or meat.

TABLE III.

Effects of the Bran and Embryo of Wheat as Protein Supplements to Wheat Flour.

Source of protein.	Protein in food.	Rat.	Initial body wt.	Gain in 4 wks.	Total intake.		Intake per gm. of gain.		Gain per gm. of protein.
					Food.	Protein.	Food.	Protein.	
Series A									
	per cent		gm.	gm.	calories*	gm.	calories*	gm.	gm.
Wheat flour	10.3	5127♂	70	40	1,310	28.5	32.8	0.71	1.40
“ gluten		5123♂	74	41	1,350	29.4	33.0	0.72	1.40
“ bran		5125♂	75	35	1,320	28.6	37.8	0.82	1.23
“ embryo + 0.1 gm. yeast daily		5126♂	70	30	1,220	26.5	40.7	0.88	1.13
Average.....							36.1	0.78	1.29
Series B.									
Wheat flour	10.3	5109♂	71	50	1,495	31.6	29.9	0.63	1.58
“ gluten		5113♂	70	35	1,255	26.8	35.8	0.77	1.31
“ bran		5106♂	69	43	1,395	29.6	32.4	0.69	1.45
“ embryo + 0.1 gm. yeast daily		5100♂	72	32	1,355	28.8	42.3	0.90	1.11
Average.....							35.1	0.75	1.36
Series C.									
Whole wheat.....	10.3	4689♂	63	44	1,310	35.2	29.5	0.80	1.25
		4669♂	60	45	1,235	33.2	27.2	0.74	1.36
		4680♂	68	39	1,100	29.6	27.9	0.76	1.32
Average.....							28.2	0.77	1.31
Series D.									
Wheat flour	10.3	5086♂	68	27	1,345	27.0	49.8	1.00	1.00
“ gluten + 0.2		5083♂	71	29	1,235	25.0	42.6	0.86	1.16
“ gm. yeast		5089♂	75	26	1,070	21.9	41.2	0.84	1.19
		5094♂	71	26	1,290	26.0	49.6	1.00	1.00
Average.....							45.8	0.93	1.09

* Estimated (protein + carbohydrates \times 4.1, fat \times 9.3 calories).

In order to compare the supplementing effect of bran and embryo protein on the growth-promoting power of the endosperm protein with that of the protein of eggs, milk, or meat, a mixture of bran and embryo in the proportion in which these occur in the whole wheat kernel was used to furnish one-third of the protein; *i.e.*, the same proportion as did the animal products in the series just discussed. The results of these experiments are shown in Table III, Series A.

From the data given in Table III we calculate that 1 gm. of protein in the ration fed to Series A caused an average gain of body weight of 1.29 gm., somewhat more than when flour and yeast supplied the protein, Series D, but decidedly less than when the flour was supplemented by comparable quantities of the animal proteins (Table II).

To learn what supplementing effect the bran and embryo proteins have on the flour proteins when used in the same proportions as they exist in the entire wheat kernel, another series of rats was fed on a diet fulfilling these conditions (Table III, Series B).

The diets supplied to Series A and B were much alike and closely resembled in protein content that used for Series C which had 92 per cent of whole wheat mixed with 5 per cent of butter fat and 3 per cent of salts, but with no added yeast. It is interesting to note that growth was at approximately the same rate in each series; namely, 1.29, 1.36, and 1.31 gm. respectively per gm. of protein eaten. The conclusion therefore seems justified that in these experiments the yeast had no appreciable supplementing effect in Series A and B.

Wheat as a Source of Water-Soluble Vitamine.

Literature.—McCollum and Davis²⁸ found that a ration containing 64 per cent of whole wheat, supplemented by casein and suitable inorganic salts, failed to promote continued growth and normal reproduction of rats unless butter fat was also added to the diet. From this they conclude that the wheat kernel contains little of the fat-soluble vitamine.

McCollum, Simmonds, and Pitz¹⁷ state that as small an amount as 15 per cent of whole wheat furnishes enough water-soluble vita-

²⁸ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615

mine for completion of growth of rats and the production of a nearly normal number of young, but not enough to enable the young to develop to the weaning age.

The same authors¹⁸ found that a commercial preparation of wheat embryo contained a moderate amount of the fat-soluble and so large an amount of the water-soluble vitamine that 2 per cent in the diet was sufficient for growth at the normal rate for several months.

The fact that the results of experiments showing the presence of the water-soluble vitamine in food products agree so well with those showing the presence of the antineuritic principle has led to the belief that the symptoms following the absence of these dietary factors from the food are due to a deficiency in the ration of intimately associated, if not identical, substances. Evidence that the antineuritic dietary constituent is unequally distributed in different parts of the seed is afforded by the newly published paper of Voegtlin, Lake, and Myers²⁹ who reach the following conclusions:

"For pigeons an exclusive diet of whole wheat or corn furnishes an adequate supply of antineuritic vitamine. The antineuritic vitamine seems to reside in the peripheral layers and the germ of these seeds, whereas the endosperm is relatively poor in this substance. If wheat and corn foods containing only a small percentage of the peripheral layers and germ of the seed are fed to pigeons and chickens exclusive of other food, polyneuritic symptoms appear on an average of three weeks after the beginning of the feeding period. The appearance of polyneuritis is preceded by a gradual loss in body weight. The birds can be relieved of their paralysis in a striking way by the oral or subcutaneous administration of a highly concentrated preparation of antineuritic vitamine derived from 'whole wheat' bread, yeast, ox liver, rice polishings, or beans. The addition of yeast (in amounts used by bakers) in the preparation of bread from highly milled flour does not prevent the appearance of polyneuritis in birds fed on this food, but prolongs slightly the period of incubation. The addition to 'highly milled' flour, or bread made from 'highly milled' flour, of a small amount of antineuritic vitamine preparation will correct this particular dietary deficiency, and will prevent the appearance of polyneuritis and the loss of body weight."

Experimental.—Adult rats fed on an otherwise adequate diet, in which the water-soluble vitamine was supplied wholly by 20 per cent of *wheat flour* (see Chart VIII, Period 1) lost weight steadily

²⁹ Voegtlin, C., Lake, G. C., and Myers, C. N., *Public Health Rep.*, 1918, xxxiii, 647, wherein some of the earlier literature on this topic is cited.

and quite rapidly. When the flour was replaced in Period 2 by an equal amount of finely ground *whole wheat* they were fully maintained for a long time. When later (in Period 3) they were returned to the wheat flour diet all lost weight, but promptly recovered in Period 4 when they were given water-soluble vitamine in the form of commercial wheat embryo, whole wheat, or yeast.

Since the impaired condition, caused by a diet deficient in water-soluble vitamine, is always followed by a loss of appetite, and the improved condition resulting from adding more of this factor to the diet is always accompanied by a marked increase in food intake, it is very difficult to compare the relative vitamine content of such food products as wheat flour and whole wheat.

An illustration is afforded by Rat 3928, Chart VIII, which, in respect to its food intake, behaved like the others. During the 2nd, 3rd, and 4th weeks of Period 1 this rat ate as much of the 20 per cent flour food per week as it ate of the 20 per cent whole wheat food during any week of Period 2. However, during the last week of Period 1 its food intake fell to about two-thirds of that previously eaten. In Period 2 its food intake rose at once, so that it ate as much as at the beginning of Period 1. Its body weight increased correspondingly and remained constant when restricted during the rest of this period to the same amount of food as that eaten in the 1st week. In Period 3 its food intake during the 1st week was the same as that in Period 2, but during the two subsequent weeks fell rapidly, accompanied by a rapid loss of body weight. Of course with declining food intake on a food containing a fixed percentage of vitamine the quantity of this factor received by the animal decreases proportionately, hence the animal quickly goes from bad to worse. Under such circumstances it is evident that comparisons based on the amount of vitamine actually consumed cannot be made. We have consequently been compelled to make this comparison on a percentage basis which, for practical purposes, furnishes information of value to those who use these products.

Growth.—In agreement with the experience of McCollum, Simmonds, and Pitz¹⁷ we have found, that provided they eat enough, *young rats* grow well when only 15 per cent of the *entire kernel* supplies the water-soluble vitamine in an otherwise adequate ration having an energy value of 5 calories per gm. This proportion

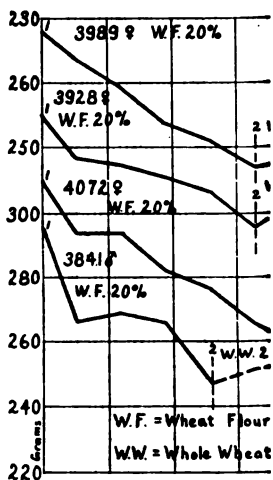


CHART VIII. *Whea*
 vitamine when either
 The failures were not d
 decline in weight when
 doses of commercial w
 Rat 3841 for which the
 failed in Period 1, was
 The composition of

Casein...
 Whole wt
 Wheat flo
 Salt mixt
 Starch...
 Butter fa
 Lard.....

* See 1



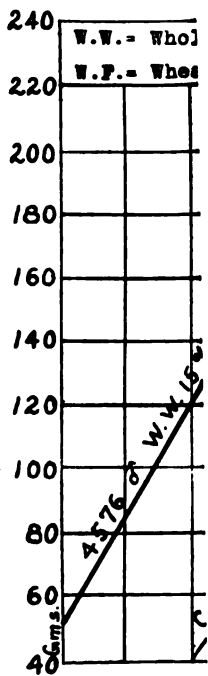
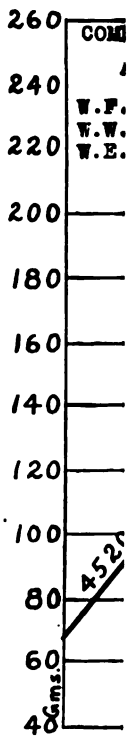


CHART IX.
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The

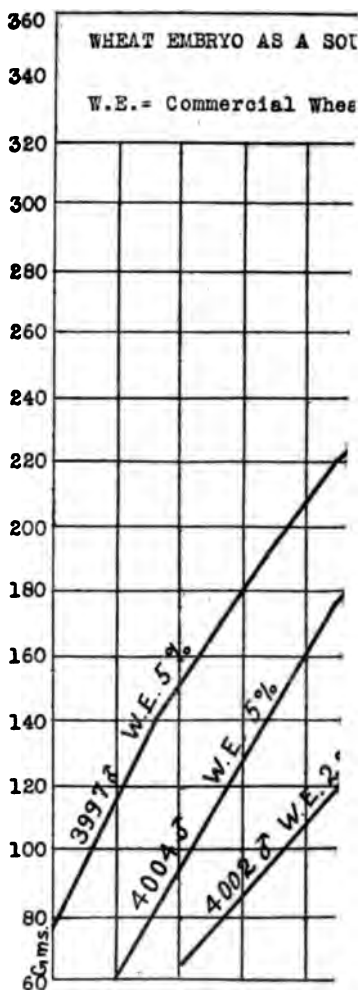


CHART XI. The effect of wheat embryo as the sole source of vitamin B₁. The composition of the feed is as follows:

Casein.....
Commercial wheat flour.....
Artificial protein.....
Salt mixture†.....
Starch.....
Butter fat.....
Lard.....

* The preparation of the feed is given in the appendix, xv, 317.

† See legend.



is evidently near the minimum required for normal growth, for with 10 per cent all of the rats soon ceased to grow, but always recovered when diets containing 20 per cent of whole wheat were fed (see Chart IX, Rats 4697, 4694, 4699).

When *wheat flour* was used as the sole source of this vitamine, even as much as 50 to 60 per cent was insufficient to promote full normal growth of young rats, while 30 per cent was barely enough to maintain them (see Chart X, Rats 4520, 4517, 4542). Rat 4520 appears to be an example of the exceptional animal, occasionally met with, which is able to grow when supplied with a much smaller amount of vitamine than is needed by the average. Rats 4570 and 4578 declined on diets containing 30 per cent of wheat flour and did not respond by growing when the flour was increased to 58 per cent. In their enfeebled condition due to the low content of vitamine in the diet, these rats ate so little food containing 58 per cent of flour that they received a relatively small quantity of the water-soluble vitamine. The amount was, therefore, much less than that obtained from the outset by Rats 4520, 4517, and 4542 on the 60 per cent food, for these were eating normally. In cases of this kind we have found that by feeding a little yeast or commercial wheat embryo in addition, the food intake is so increased that after 4 or 5 days the yeast can be omitted and thereafter good growth can be made on the former diets containing minimal amounts of vitamine. We conclude that 60 per cent of wheat flour furnishes less than the optimum quantity of the water-soluble vitamine.

Of the *commercial embryo meal* used in our experiments 2 per cent was a smaller quantity than we have found to be adequate to promote normal growth. On an average food intake of 50 gm. per week the minimal quantity would therefore be more than 1 gm. weekly (see Chart XI).

In testing *commercial wheat bran* as a source of water-soluble vitamine it was found that when 5 per cent was used as the sole source of this vitamine in an otherwise adequate ration, *young* rats soon ceased to grow (see Chart XII). *Adult* rats, on the other hand, were not even maintained on similar rations (see Chart XIII). A small amount of commercial embryo fed each day apart from the food mixture containing bran and in addition to it, induced prompt growth of young, or recovery of adult rats (see

Charts XII and XIII). The good effect of the added embryo ceased when the latter was discontinued. Without the added vitamin none of these rats would eat enough food. When 55 per cent of commercial bran, equal to 45.5 per cent of *pure* bran, was fed, as in the experiment where bran was used as the sole source

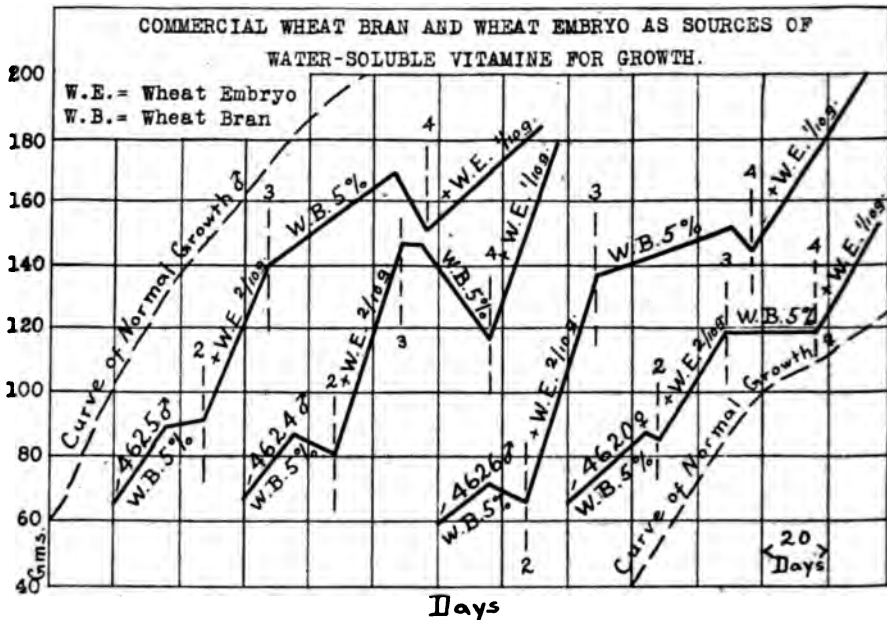


CHART XII. Showing the failure of wheat bran (W.B.) to supply enough water-soluble vitamin, Periods 1 and 3, in contrast with the rapid gain in weight, in Periods 2 and 4, caused by 0.1 to 0.2 gm. of commercial wheat embryo (W.E.) fed each day separately and in addition to the same diet as was furnished in Period 1.

The composition of the food mixture was as follows:

	per cent
Meat residue*	19
Commercial wheat bran	5
Salt mixture†	4
Starch	48
Butter fat	9
Lard	15

* See Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 313.

† See legend, Chart I.

of protein, the addition of wheat embryo had no beneficial effect, showing that the commercial bran used contained some water-soluble vitamine.

Our numerous experiments with commercial wheat embryo meal at first led us to conclude that nearly, if not quite all of the vitamine present in the wheat kernel is contained in the embryo. Comparisons of the quantities of each of the different commercial parts of this seed, however, which, when used alone to supply vitamine, sufficed to promote normal growth, showed that this could not be the case. Accordingly we isolated the embryos from the kernels, taking care to obtain them in the purest possible condition. To our surprise no growth whatever resulted when 22 mg. of the pure embryo were given daily. This amount is equal to

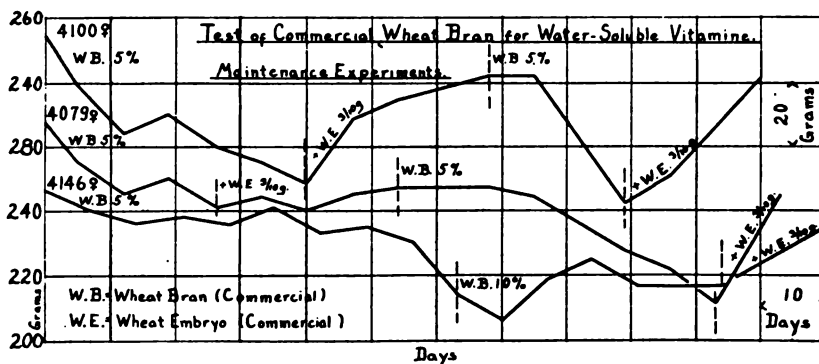


CHART XIII. Adult rats failed to be maintained for any length of time when the water-soluble vitamine was furnished solely by 5 per cent of a commercial preparation of wheat bran (W.B.). Improvement invariably followed the feeding of small doses of commercial wheat embryo (W.E.).

The composition of the food mixtures was as follows:

	per cent
Meat residue*	19
Wheat bran	5-10
Salt mixture†	4
Starch	48-43
Butter fat	9
Lard	15

* See Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 313.

† See legend, Chart I.

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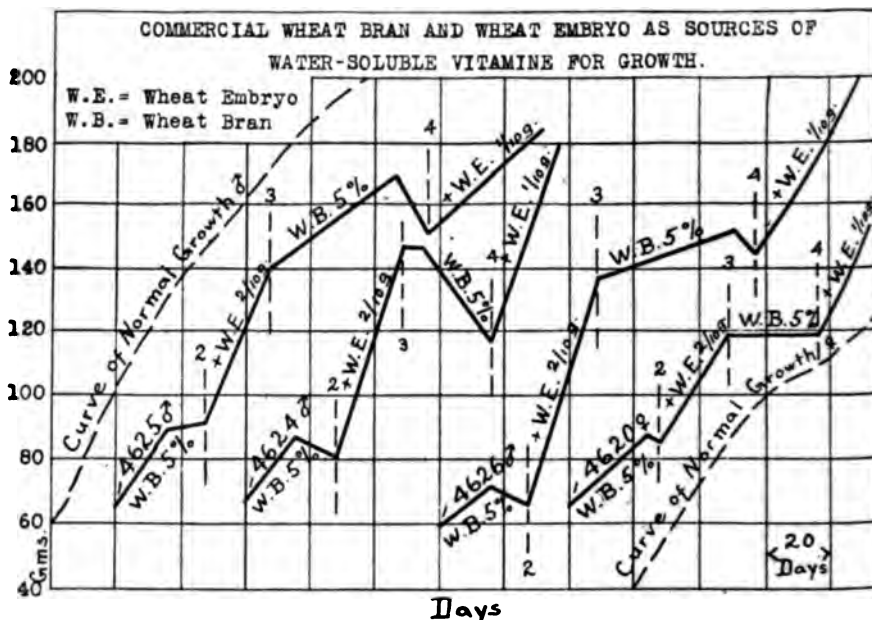


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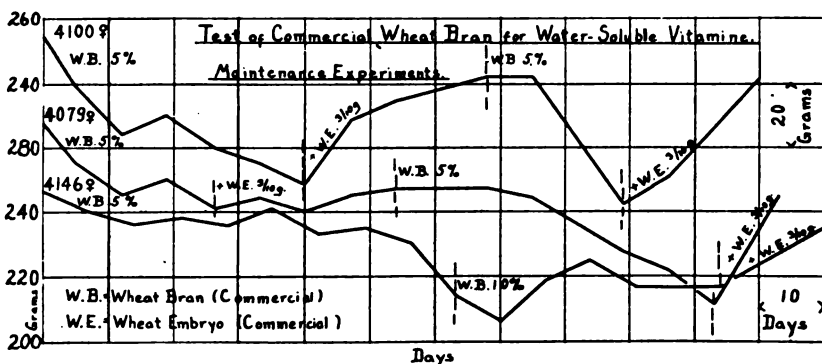


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† See legend, Chart I.

the embryo in the 15 per cent of whole wheat used in the experiments shown in Chart IX, assuming an average food intake of 70 gm. per week. The vitamine thus supplied was sufficient to promote practically normal growth. When the pure embryo was given in successively increasing amounts up to 150 mg. the result was the same. Although none of these rats gained appreciably in weight, none of them declined. Thus one rat, which weighed 65 gm. at the beginning of the experiment, weighed only 76 gm. after 71 days, a gain of only 11 gm., instead of 115 gm. which it should have made under normal conditions. Although growth practically failed, maintenance was perfect. Other experiments confirmed this result. On the same diet, with no added source of vitamine, a prompt and rapid loss of weight always occurred.

Since Rats 3997 and 4004 (see Chart XI) when supplied with commercial embryo meal in quantity equal to 180 mg. daily of the pure embryo grew normally, while Rat 4002 which had the equivalent of only 60 mg. daily of pure embryo grew only a little less rapidly, we must conclude that the 150 mg. of the pure isolated embryos given in this experiment should have caused good growth if the water-soluble vitamine of the wheat kernel is a single substance present only in the embryo.

To determine whether the embryo actually contributes anything to the vitamine content of the entire kernel we removed the embryos from a quantity of wheat, taking care to leave as little as possible adhering to the seed, and we believe that at the most only very small traces escaped removal. When these embryo-free kernels were fed as the sole source of water-soluble vitamine they proved quite as efficient in promoting growth as did the entire wheat kernel.

We next cut off about one-quarter of the embryo-free grain at the embryo end of the seed and fed each part separately as a source of vitamine. In comparable quantities the end near the embryo was more efficient than the remainder of the seed though the latter was by no means devoid of activity. Combining pure embryo with either of these parts of the kernel made no apparent difference in the results.

The rats fed on diets in which the pure embryo alone supplied vitamine were well maintained for many weeks without evidence of impaired vigor; but no appreciable growth was made. On the

other hand the remainder of the seed was capable of promoting practically normal growth. These observations raise a question as to whether the water-soluble vitamine is a single substance or a mixture of two or more.

It thus appears that the vitamine is located in the endosperm, but is not uniformly distributed throughout it. This conclusion is not at variance with our experiments with wheat flour or commercial embryo meal nor with Voegtlin, Lake, and Myers' discovery that patent flour is practically free from vitamine. In making flour by gradual reduction the grain is broken into successively smaller particles, a bolting process being interposed between the breaks. The finer particles, which represent the more friable part of the endosperm, are thus sifted out and the larger and harder particles which remain on the sieves are ground into patent flour. If the vitamine is in fact concentrated in the softer parts of the endosperm we should expect the patent flour to be nearly free therefrom and the lower grades to be correspondingly richer therein. The high vitamine activity of the commercial embryo meal may be caused by a considerable proportion of the softer parts of the endosperm adjacent to the embryo which in the milling process is removed together with that part of the seed. Voegtlin, Lake, and Myers say that "the antineuritic vitamine seems to reside in the peripheral layers and the germ of these seeds, whereas the endosperm is relatively poor in this substance."

Our experiments appear to make it certain that if the vitamine is a single substance needed for adequate nutrition, it must therefore be a constituent of the endosperm.

Alleged Toxicity of Wheat.

In a series of papers from the Wisconsin Agricultural Experiment Station³⁰ upon the effects of rations derived from restricted plant

³⁰ Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239. Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Proc. Nat. Acad. Sc.*, 1917, iii, 374; *J. Agric. Research*, 1917, x, 175. Hart, E. B., Steenbock, H., and Humphrey, G. C., *Univ. Wisconsin Agric. Exp. Sta., Bull.* 287, 1918.

sources it has repeatedly been alleged that wheat contains a toxic substance. The "inherent toxicity" of the wheat grains is said to reside particularly in the embryo. It is reported that addition of a large amount of wheat embryo frequently produced an early abortion in cattle. Diets high in their wheat content were regarded as particularly injurious to the progeny. The toxicity is stated to assert itself even in the presence of all the recognized factors for growth. In the case of swine,³¹ for example, the investigators state:

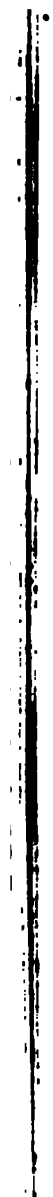
"Only in the presence of very liberal quantities of all these factors can the effect of the toxicity be overcome. This toxicity manifests its action by producing important histological changes in the nervous system of the animal, not unlike those recorded for beri-beri. No one important factor for growth, such as better proteins, salts or fat-soluble A, appears able to act as a complete corrective for this toxicity."

Doubts respecting the validity of such conclusions have already been referred to. We have succeeded in growing many rats over a period of a year when whole wheat supplied the protein and water-soluble vitamine. Females have borne litters containing an average number of young (Chart II). Three litters of these have survived, but all were undersized though apparently vigorous. This failure of rats fed on 92 per cent of whole wheat to produce young of normal size is not at variance with the observations of the Wisconsin investigators.

When commercial wheat embryo to the extent of 50 per cent of the diet supplied all of the protein and water-soluble vitamine during nearly a year the rats grew well as a rule and bore several litters of young (Chart IV). Nearly one-half of these young either died or were eaten. This relatively high mortality is, however, not much if any greater than we have encountered in raising rats under experimental conditions. Inasmuch as the surviving young, kept on the same diet, are growing at a nearly normal rate (see Chart XIV) we do not regard the mortality as evidence of toxicity in this diet. The females might have bred more frequently if they had been mated more often.

In view of the successful outcome of these experiments with diets containing such a large proportion of the embryo, in which the

³¹ Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239.



"poisonous" factor has been assumed to be concentrated, we are not prepared as yet to accept the view that the wheat kernel contains a "toxic" substance. The failure of the rats on the 92 per cent whole wheat diet to reproduce quite normally may be attributed as well to abnormal and inappropriate proportions of otherwise desirable food factors as to anything that may be properly designated as poisonous. It is unfortunate that a prejudice has been created against wheat before convincing evidence of the existence of a poison has been thoroughly substantiated.

To What Extent Should Wheat Be Milled?

The economical use of the wheat kernel demands that its different parts be fed under conditions such as will ensure their most complete utilization. Heretofore the discussion of this problem has centered chiefly about the digestibility of the nutrients furnished by highly milled flour and that made by grinding the entire seed.²² Recognition of the wide differences in nutritive value of proteins from different sources, as well as the need of those accessory food substances known as vitamins, has put in a new light the question as to the degree to which the wheat kernel should be milled. Before reaching a final conclusion some of the results of the experiments described in this paper deserve consideration.

It has been shown that flour, when used as the sole source of protein, is inferior to other foods in maintaining adult rats, and especially in promoting the growth of young; that when combined with about one-third of their weight of the proteins furnished by eggs, milk, or meat, the wheat proteins are so greatly enhanced in value that flour is thus used most advantageously; that the content of water-soluble vitamin in flour as usually made is relatively small; that the proteins of the bran and embryo are superior in nutritive value to those of the endosperm, from which white flour is made; and that commercial preparations of the embryo contain a relatively large amount of the water-soluble vitamin.

Since by far the greater part of the flour used in this country is eaten in combination with those food products which successfully supplement the nutritive deficiencies of its proteins to a far greater

²² Various aspects of the question have been presented anew by Snyder, H., *Science*, 1918, xlvii, 429; and Dutcher, R. A., *ibid.*, 1918, xlvii, 228.

extent than do the bran and embryo of the entire kernel, no practical advantage on this score can be expected by converting the entire grain into flour. It is true that commercial embryo meal is rich in water-soluble vitamins and that patent flour is much poorer in this food factor than flour containing all parts of the grain. Nevertheless it is almost certain from what has been learned of the vitamin content of the other food products commonly eaten with bread that the vitamin deficiencies of patent flour are made good thereby. That this is the case is also supported by the fact that the great majority of people who live on the dietaries which prevail in this country show no evidence of ill effects which indicate deficiencies in the amount of vitamins they are receiving.

Furthermore, since the nutrients of bran are poorly utilized by mankind a waste of food, though probably not large, results when the bran is included in the flour. Although the constituents of the embryo have a high nutritive value the embryo forms so small a part of the entire kernel, and so impairs the keeping qualities of the flour, that probably the nutritive advantages of including the embryo in the flour are more than counterbalanced by the practical disadvantages.³³

We therefore feel justified in the conclusion that, except in special cases, little can be gained by including bran and embryo in the flour when this is used under the conditions prevailing in this country.

When we consider that the rations of farm animals are generally of such a character that protein supplements are needed to increase the proportion, as well as to supplement the chemical deficiencies of their protein, the question arises whether it is more profitable to feed the by-products of milling to animals or to man. The superior supplementing value of the animal proteins produced by the former procedure when used with wheat flour, compared with that of the proteins of bran, as we have shown in this paper, certainly reduces the loss that has heretofore been assumed to result from this practice.

Experience has shown that the proteins of bran are not readily digested by man and that intestinal disturbances are not infre-

³³ Taylor, A. E., *War bread*, New York, 1918.

quently attributed to the presence of such amounts of bran as are present in flour made by grinding the entire seed. Furthermore agriculturists are convinced that wheat bran is a valuable source of protein in the ration of farm animals. With this conviction the results of our experiments agree.

Armsby³⁴ has recently demonstrated that coarse fodders, unfit for human food, provide sufficient nutriment for the maintenance of farm animals, and that the energy of the concentrated cereal products supplied in addition thereto is utilized to an extent of 60 to 85 per cent for the production of human food. This view, in conjunction with the fact that animal proteins thus produced, when eaten together with flour, render a smaller quantity capable of supplying the protein requirements of the young, and perhaps also those of adults, further supports Lusk's conclusion, which was based solely on Armsby's data, that "it is evident that food is best conserved for man when edible grains are taken to the miller and the bran is used in meat production."³⁵

We can expect, therefore, that the by-products of milling will be better utilized on the farm than on the table. It is difficult to establish what their relative value is when used as human food or fed to farm animals. Many other factors than those of nutrition enter when this question is dealt with in practice; but apart from these, it has been made plain by this investigation, that one has heretofore not received proper consideration. Bran and embryo together form about 17 per cent of the wheat kernel; the endosperm from which flour is made forms the remaining 83 per cent. About one-half as much flour eaten with the animal products, in such proportion that one-third of the protein is furnished by the latter, is capable of satisfying the *protein* requirements as when flour alone furnishes all of the protein. If, therefore, about 80 per cent of the wheat kernel can be so improved in nutritive value by adding animal products to the diet that a much smaller amount of flour will satisfy the protein needs of nutrition, it may well be that the use of the by-products of milling for the production of meat, milk, or eggs will result in a greater economy in the use of flour than if these were used directly for human food.

³⁴ Armsby, H. P., The conservation of food energy, Philadelphia, 1918.

³⁵ Lusk, G., *Science*, 1918, xlviii, 447.

To what extent the consumption of wheat flour may be reduced by additions of animal proteins to the ration will depend largely on individual requirements. Adults engaged in hard labor, whose calorific needs are high, can probably meet their demand for protein by the small percentage of relatively poor protein which flour supplies. On the other hand it may be that those of sedentary habits, as well as growing children, can be better and more economically fed when a suitable addition of eggs, milk, or meat is made to the bread ration.

In view of the foregoing considerations the aim of the miller should be to effect such a separation of the other parts of the wheat kernel from the endosperm as will lead to a minimal transfer of the latter into offal. Every grade of flour which is made with a loss of endosperm into milling by-products represents a loss of human nutrients.

Whenever bread made from highly milled flour forms an unduly large proportion of the diet of children the vitamin deficiencies which the experiments described in this paper make so evident, may lead to malnutrition. In this country there is relatively little occasion to be concerned on this score, because, as we have already pointed out, the food habits of our people are such as to make cases of this kind comparatively rare.

That a real danger may be incurred by a too exclusive use of bread made from highly milled wheat is shown by numerous cases referred to by Chick and Hume.³⁶ They have reported that among groups of people living on restricted diets in which bread made from patent flour formed a large proportion of the total ration, beri-beri was very common; whereas people living on similar diets, but with bread made from the entire kernel replacing that made from patent flour, were rarely affected.

SUMMARY.

The quantity of protein furnished by the *entire* wheat kernel which is necessary for continued maintenance of adult rats is greater than that required when proteins of milk or of various

³⁶ Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1916-17, x, 141; *Proc. Roy. Soc. London, Series B*, 1917, xc, 44, 60.

other food products are fed. For the normal growth of the young this difference is much more pronounced.

The "crude protein" of the commercial embryo is superior to that of the entire kernel or of the endosperm in maintaining adults. For satisfying the needs of growing rats the embryo proteins are somewhat more efficient than are those of the entire kernel and much more efficient than are those of the endosperm.

The "crude protein" of commercial bran if eaten in sufficient quantity is somewhat superior to that of commercial embryo and decidedly superior to that of the endosperm in promoting the growth of young rats.

For maintaining adults the proteins of the endosperm are adequate; they are inadequate for growth. Additions of meat, milk, or eggs to wheat flour so greatly enhance the value of the protein for growth that a great economy in consumption of protein results. Under the ordinary human dietary conditions the proteins of wheat are advantageously employed.

Commercial wheat embryo is rich in the water-soluble vitamine. Commercial bran and flour contain much less of this important food factor. The *pure embryo*, carefully separated from all the other parts of the seed and used as the sole source of vitamine suffices to maintain young rats, but fails to promote their growth, even when supplied in quantities equal to the amount of pure embryo contained in such quantities of the commercial embryo as were sufficient to promote full normal growth. Wheat kernels from which the embryo has been carefully removed are still rich in water-soluble vitamine.

Rats fed for 1 year from the time of weaning on diets containing 92 per cent of wheat or 50 per cent of commercial wheat embryo reached full maturity without giving any evidence that wheat contains a toxic substance.

The relation of these experiments to the problems raised by modern milling methods and the degree to which it is desirable to mill flour for general use has been discussed.

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